DOCTOR OF PHILOSOPHY

Characterisation of candidate effector proteins from barley pathogen Rhynchosporium commune

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Characterisation of candidate effector proteins from barley pathogen *Rhynchosporium commune*

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List of Abbreviations

ACIK1	Avr9/Cf-9 induced kinase 1
ACRE	Avr9/Cf-9 Rapidly Elicited
AOS	Allene oxide synthase *(*AOS*)*
ATMT	*Agrobacterium tumefaciens*-mediated transformation
Avr	Avirulence
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
Bir1-1	Bak1-interacting receptor-like kinase 1,1
BLAST	Basic Local Alignment Search Tool
BR	Brassinosteroid
BRI1	Brassinosteroid-Insensitive1
BSMV	Barley stripe mosaic virus
Cas9	CRISPR associated protein 9
CBEL	Cellulose-Binding Elicitor Lectin
CBS	Center for biological sequence analysis
cDNA	Coding DNA
CEBiP	Chitin elicitor-binding protein
CERK1	Chitin elicitor receptor kinase 1
CF-4/ CF-9	*Cladosporium fulvum*-4/9
CMPG1	Cys, Met, Pro, and Gly protein 1
Col-0	Columbia-0
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRN	Crinkling and Necrosis
CS	Culture supernatant
CSP	cold shock protein
Cv	Cultivar
DAMPs	Damage-associated molecular patterns
DMI	Demethylation inhibitor
DNA	Deoxyribonucleic acid
DPI	Days post inoculation
DSB	Double-Strand DNA Break
DTT	DiThioThreitol
EB	Elution buffer

EDTA	Ethylene Diamine Triacetic Acid
EFR	EF-Tu receptor
EF-Tu	Elongation factor Tu
eGFP	Enhanced GFP
EPIC	Extracellular Cystatin-like Protease Inhibitor
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EV	Empty vector
FGSC	Fungal Genetics Stock Center
FLARE	Flagellin Rapidly Elicited genes
Flg22	22 amino acid motif from bacterial Flagellin
FLS2/3	Flagellin sensing 2/3
GC	Germinated conidia
gDNA	genomic DNA
GFP	Green Fluorescent Protein
GH12	Glycoside hydrolase family 12
gtPCR	genotyping PCR
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HG	Homogalacturonan
HM1	*Helminthosporium maydis* 1
Hop	Hrp-dependent outer protein
hph	Hygromycin-B-Phosphotransferase
HR	Hypersensitive Response
HRP	Horse Radish Peroxidase
HsbA	Hydrophobic surface binding protein A
iLOV	improved light, oxygen or voltage
INF1	Infestin 1
ISR	Induced systemic resistance
JA	Jasmonic acid
LB	Luria-Bertani
LeFLS2	Tomato flagellin receptor
LF	Left flaking region
LPS	Lipopolysaccharides
LRK1	Lectin receptor kinase 1
LRR	Leucine-rich repeat

LRR-RLKs	Leucine-rich repeat receptor-like protein kinases
LRR-RLPs	Leucine-rich-repeat receptor protein
LysM	Lysin Motifs
MAMP	Microbe associated molecular pattern
MAPK	Mitogen-activated protein kinase
MAPKKKε	*M*itogen-activated protein kinase kinase kinase epsilon
MAT	Mating-type
MBC	Methyl benzimidazole carbamate
MEGA6	Molecular Evolutionary Genetics Analysis Version 6
MEK2	Mitogen-activated Protein Kinase Kinase 2
mRFP	monomeric RFP
mRNA	messenger RNA
NB-LRR	Nucleotide Binding and Leucine-Rich Repeat
NCBI	National Center for Biotechnology Information
NHEJ	Non-Homologous end joining
NIP	Necrosis-inducing proteins
NLP	Necrosis and ethylene-inducing peptide 1-like protein
NPR1	Nonexpressor of Pathogenesis-Related protein1
OG	Oligogalacturonide
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDB	Potato Dextrose Broth
PEG	Polyethylene glycol
PEP	Plant Elicitor Peptide
PG	Endopolygalacturonase
PGN	Peptidoglycan
PR	Pathogenesis-related
PR1	Pathogenesis-related gene 1
PRR	Pattern recognition receptor
PTI	PAMP triggered immunity
PTI5	Pathogenesis-related genes transcriptional activator
PTP	Protein tyrosine phosphatase
PVPP	Polyvinylpolypyrrolidone

PVX	Potato virus X
qRT-PCR	Quantitative Reverse-Transcriptase PCR
QTL	Quantitative trait loci
R	Resistance
RBOHB	Respiratory burst oxidase homolog protein B
RBPG1	Responsiveness to Botrytis Polygalacturonase1
Rc2	*Rhynchosporium commune* 2
RcCDI1	*Rhynchosporium commune* inducing 1
Rcr3	Required for *C. fulvum* resistance 3
RF	Right flaking region
RFP	Red Fluorescent Protein
RIN4	Rpm1-Interacting protein 4
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA	Ribonucleic Acid
RNAi	RNA interference
RNP-1	RNA-binding Protein motif-1
ROS	Reactive oxygen species
Rpm1	Resistance to *Pseudomonas syringae* pv Maculicola 1
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic Acid
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDW	Sterile distilled water
SERK	Somatic embryogenesis receptor kinase
SGT1	Suppressor of G2 allele of Skp1
SIPK	Salicylic acid-induced protein kinase
SNPs	Single nucleotide polymorphisms
SOBIR1	Suppressor of BIR1-1
SP cluster	Clustered serine-proline residues
SP	Signal peptide
SYBR	Synergy Brands, Inc.
T3SS	Type III secretion system
TBS	Tris-Buffered Saline

T-DNA	Transfer DNA
TMV	Tobacco mosaic virus
TPR	Tetratricopeptide repeat
TRV	Tobacco rattle virus
UV	Ultraviolet
VIGS	Virus induced gene silencing
VOX	Virus over expression
WIPK	Wounding-induced protein kinase
YPD	Yeast extract-peptone-dextrose
YRC	Yeast recombination cloning

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I would like to dedicate this work to my family…

Declarations

The results presented in this PhD thesis are from investigations conducted by myself. Any work that is not my own is clearly identified with the appropriate references and publications. I hereby declare that I am the author of this work and it has not been previously accepted for any higher degree.

Barbara Franco Orozco

We certify that Barbara Franco Orozco has fulfilled the relevant ordinance and regulations of the University Court and is qualified to submit this thesis for the degree of Doctor of Philosophy.

Dr. Anna Avrova, The James Hutton Institute

Abstract

Rhynchosporium commune causes leaf scald, one of the most devastating diseases of barley. It leads to significant yield losses, grain quality can also be affected, resulting in substantial losses for barley producers. Disease management strategies mainly rely on the use of fungicides and resistant varieties but *R. commune* populations can change rapidly, overcoming barley resistance and some fungicides. This study aimed for the characterisation of molecules released by *R. commune* during the interaction with its host barley. Analysis of transcripts produced by *R. commune* during infection of barley plants is a valuable resource for identification of pathogenicity factors. Sequencing of the interaction transcriptome from an early time point during barley infection with *R. commune* revealed three abundant transcripts coding for small secreted fungal proteins called RcCDI1, Rc2 and Rsu3_07158, this last one encoding for a hydrophobic surface binding protein. They are most highly up-regulated early during infection. RcCDI1 and its homologs from different fungal species exhibit PAMP activity. RcCDI1-triggered cell death was shown to require SGT1, SOBIR1 and BAK1 but was not suppressed by PiAVR3a, PexRD2, PexRD27 and AvrPto. Transient expression of truncated versions of RcCDI1 protein in *N. benthamiana* indicated that the N- and C-terminal domains of RcCDI1 are required for the induction of cell death. Identification of the plant receptor involved in RcCDI1 recognition in dicots will provide a valuable resource for engineering non-host resistance in monocots. The overexpression of *Rc2* allele from *R. commune* strain L2A in the barley landrace SLB-10-009 induced necrotic lesions, indicating a potential recognition of the candidate effector Rc2. Virulence tests were performed to see whether single nucleotide polymorphisms (SNPs) in the gene sequence of *Rc2* correlate with virulence/avirulence of the *R. commune* strains in the barley line SLB 10-009. Finally, targeted gene disruption was used as an approach to characterise RcCDI1, Rc2 and Rsu3_07158 candidate effectors.

Chapter 1. General introduction

1.1 Crop pathogens: A threat to agricultural production worldwide

In the history of agriculture, ancient humans dedicated to hunting and gathering as survival activities migrated to different locations before farming began (Zvelebil & Pluciennik, 2003). The transition from a hunter-gatherer behaviour to a more settled life-style led to the domestication of plants. Some of the crops had higher yields than others and they were expanded rapidly as basic food for many societies (Diamond, 2002). However, the spread of agriculture to different regions also came with the spread of important pathogens leading to an increase in crops losses caused by diseases (Agrios, 2005; Sharma *et al.*, 2013). Many people died from starvation, while both causes of diseases and any control practices were completely unknown.

At present plant pathogens continue to devastating crops. A very well-known example of the importance of plant pathogens is illustrated by the Irish potato famine which occurred in 1845 and was caused by the potato pathogen *Phytophthora infestans*. This dramatic event did not just happen in Ireland, but also in other European countries (Zadoks, 2008). Due to this famine approx. 600,000 people died and during the period of 1846-1851 approx. 1,300,000 Irish people emigrated (Zadoks, 2008). Another example of a devastating pathogen leading to losses worldwide is *Fusarium oxysporum*, the causal agent of the Panama disease of banana affecting susceptible Gros Michel cultivar (Ploetz, 2000). Losses of 30,000 hectares were reported in the Ulua Valley of Honduras, for example, in the 20 years period between 1940 and 1960 (Ploetz, 2000). As a control strategy, susceptible cultivars were replaced by the resistant cultivar from the Cavendish subgroup but in some areas they also became equally affected (Ploetz and Pegg, 2000). Due to the huge economic impact of these crop diseases, scientists started looking for their causal agents, for an improved understanding of plant immunity that could lead to better resistance strategies.

Heinrich Anton de Bary made an extensive study of plant pathogens summarized in his book from 1863 named "*Recherchessur le développement de quelques champignons parasites à thalle subcuticulaire*". This book provided the first insights into fungal identification, increasing the knowledge about plant pathogens. The thirst for knowledge continued with research being conducted over the years in diverse areas of plant-pathogen interactions. At present, many different plant pathogens are of high economic importance worldwide because of the devastating yield losses. Examples of these pathogens include: *Magnaporthe oryzae* causing losses of up to 30% of the annual rice yield (Talbot, 2003); *Zymoseptoria tritici* and *Fusarium sp*. responsible respectively for losses of 30%-50% (HGCA, 2012) and up to 30% (AHDB, 2016), of wheat in the UK; *P. infestans* with total losses in potato and tomato (crop losses and fungicide control) of about US$6.7 billion per annum (Haverkort *et al.*, 2008); *P. sojae* with annual losses of US$1-2 billion in soybean worldwide (Tyler, 2007); and *Botrytis cinerea* (Dean *et al.*, 2012) for which yield losses can exceed 50% in strawberry (Ellis *et al*., 1982).

1.2 Molecular plant-pathogen interactions

1.2.1 Plant immune system

Through the years, plants have been facing the attack from a broad range of microbes, considered a threat to agriculture worldwide. Plants have found ways to defend themselves, giving an indication of the presence of a plant immune system. Plant breeders have shown differences in the levels of susceptibility of the plants to the infection by pathogens through the years. The genetic inheritance studies conducted by Gregor Mendel in the 1800s led to the recognition by plant breeders that plant resistance to diseases was often inherited as a dominant or semi-dominant trait (Keen, 1990).

The concept of plant disease resistance and the gene for gene interaction model were first described by H.H. Flor in the 1940s with the study of the flax interaction with the rust causing

pathogen *Melampsora lini* (Flor, 1955, 1971). The theoretical basis of this model states that for each dominant gene conferring resistance in the host, there is a corresponding dominant pathogenicity gene conferring avirulence in the parasite (Flor, 1955, 1971). This important finding suggests the presence of a large number of avirulence (Avr) genes encoding proteins that can be recognized by host resistance (R) proteins triggering a set of defence responses in plants usually leading to a localized host cell death called hypersensitive response (HR) (Dangl, 1996; Dangl & Jones, 2001). Alteration in any of these genes by the plant or pathogen often leads to a compatible interaction (host susceptibility). The term hypersensitive was first described by Stakman, (1915), with HR responses being observed in different cereal crops including oat, wheat and barley upon infection with the rust fungus *Puccinia graminis*. The first avirulence gene isolated was a*vrA* from from *Pseudomonas syringae* pv. *glycinea* (Staskawicz *et al.*, 1984) and the first plant resistance gene isolated was *HM1* from corn (Johal & Briggs, 1992).

Many different Avr and R proteins have been characterized through the years providing a better understanding of the plant-microbe interactions (Dangl & Jones, 2001). Very well-known examples of R proteins recognising pathogen effectors include the potato R protein R3a, recognising the *P. infestans* effector Avr3a on its avirulent isoform Avr3aKI (Armstrong *et al.*, 2005); the tomato R protein Cf-4 mediating the recognition of the *C. fulvum* effector protein Avr4 (Jones et al., 1993; Joosten *et al.*, 1994; Stergiopoulos & de Wit, 2009) and the recognition of AvrPto from *Pseudomonas syringae* pv tomato by receptor kinase Pto in tomato (Shan, 2000)

Besides the fact that many R-Avr protein interactions have been described, no physical interaction was observed for some of these combinations, suggesting that their perception is indirect (Van der Hoorn & Kamoun, 2008). It also suggested that diverse pathogen effectors can be recognised by a single R protein (Dangl & Jones, 2001). These facts were explained by

the “guard hypothesis” in which R proteins guard a host target and defense response is triggered by the modification of this “guardee” protein (Van Der Biezen & Jones, 1998; Jones & Dangl, 2006; Liu *et al.*, 2007). For example, the *Arabidopsis thaliana* R proteins Rpm1 and RPS2 interact with the Arabidopsis R protein RIN4 and *P. syringae* effectors AvrRpm1 and AvrB (Mackey *et al.*, 2002). AvrRPm1 and AvrB induce the phosphorylation of RIN4 protein to suppress defence responses. RPM1 and RPS2 guarding the RIN4 protein recognize this modification and induce defence responses in Arabidopsis (Mackey *et al.*, 2002).

1.2.2 Pathogen associated molecular patterns (PAMPs)

Plants are submitted to a broad range of biotic and abiotic factors that can interfere with their health. Abiotic stress involves nutrients and water deficiency, heat or cold stress, high salinity, UV-irradiation and others (Wang *et al.*, 2003). Biotic stress involves the infection by pathogens, including viruses, bacteria, fungi and oomycetes, and pests (Dangl & Jones, 2001). Plants and pathogens are in a constant battle: plants activating a series of immune responses to fend off infection and pathogens trying to suppress or avoid these activated plant defence responses. Initial plant layers of defence involve natural structural barriers like cell walls, waxes and cuticles which limit pathogen invasion (Jones & Dangl, 2006). Inducible defence responses follow these structural barriers and consist initially in the detection of microbe or pathogen associated molecular patterns (PAMPs) triggering the very well-known PAMP triggered immunity (PTI) (Jones & Dangl, 2006). Because these specific conserved molecules are not just derived from pathogen species, but are also present in beneficial microbes, they were referred to as MAMPs instead (Ausubel, 2005). Very well-known examples of PAMPs are bacterial flagellin, the protein subunit building up the filament of the bacterial flagellum (Felix *et al.*, 1999), elongation factor Tu (EF–Tu), the most abundant bacterial protein (Kunze *et al.*, 2004), peptidoglycan (PGN) (Gust *et al.*, 2007) and lipopolysaccharides (LPS) (Zeidler *et al.*, 2004) as bacterial cell wall components, chitin from fungal cell walls (Kohler *et al.*, 2016), ergosterol, the most predominant sterol found in fungal cell membranes (Klemptner *et*

al., 2014), β-glucans (Fesel & Zuccaro, 2016) and elicitins (Kamoun *et al.*, 1997) from oomycetes. Besides PAMPs, plants are also able to recognize damage-associated molecular patterns (DAMPs), which are plant degradation products released during pathogen invasion processes (Boller & Felix, 2009). An example of DAMPs are oligogalacturonides (OGs), oligomers of α-1,4-linked galacturonic acid, which are fragments of pectin released during pathogen infection activating plant immunity (Benedetti *et al.*, 2015).

PAMPs are usually recognised due to the presence of highly conserved epitopes essential for the induction of defence responses. The best known example of such an epitope is flg22, a 22-amino acid peptide corresponding to the most conserved domain of bacterial flagellin (Gómez-Gómez *et al.*, 1999). The core part of flg22, flg15, was shown to be the smallest part of flagellin recognized by the plant cells, although at higher concentration compared to flg22 (Felix *et al.*, 1999). The complete EF-Tu sequence is highly conserved; however, the whole EF-Tu protein is not required for the induction of PTI (Kunze *et al.*, 2004). Using protein truncations, the PAMP activity of EF-Tu was localised to the acetylated N-terminal region with the peptide comprising the first 26 amino acid residues, termed elf26 (Kunze *et al.*, 2004). A shorter peptide, elf18, comprising the acetyl group and the first 18 N-terminal amino acids, was shown to be the smallest part of EF-Tu as active as elf26. Both elf26 and elf18 were shown to induce an oxidative burst, biosynthesis of ethylene and resistance to infection caused by the pathogenic bacterium *P. syringae* pv tomato DC3000 (Pst DC3000) in Arabidopsis plants (Kunze *et al.*, 2004). The peptide elf16 showed significantly lower activity, with only residual activity found with elf14. While the peptide elf12 is inactive as elicitor, it was shown to act as a specific antagonist for EF-Tu–related elicitors (Kunze *et al.*, 2004).

Another well characterised bacterial PAMP is the cold shock protein (CSP) which is highly induced at very low temperatures. It was initially identified in the bacterium *Staphylococcus aureus* and leads to the elicitation of defence responses in Solanaceae mediated by the

recognition of the conserved RNA-binding motif RNP-1 (Felix & Boller, 2003). Endopolygalacturonases (PGs) from fungal pathogens are also considered to be PAMPs and play an important role as virulence factors. They hydrolyse the homogalacturonan (HG) region of pectin which is a major component of the plant cell wall (Zhang *et al.*, 2014). Necrosis and ethylene-inducing peptide 1-like proteins (NLPs) are PAMPs produced by several species including bacteria, fungi and oomycetes (Oome & Van den Ackerveken, 2014). The first NLP was identified from the culture filtrate of *Fusarium oxysporum* (Bailey, 1995). It has been found that NLPs are capable of inducing necrosis and ethylene production in dicot plants, but monocots are completely insensitive (Bailey, 1995; Fellbrich *et al.*, 2002). All NLP proteins have in common the conserved heptapeptide motif (GHRHDWE) and conserved cysteine residues (Ottmann *et al.*, 2009). Most NLPs have a 20 amino acid region (nlp20), which induces defence responses in plants of Brassicaceae family (Böhm *et al.*, 2014; Oome *et al.*, 2014).

1.2.3 Pattern recognition receptors (PRRs)

PAMPs are recognised by cell surface receptors known as pattern recognition receptors (PRRs). They can be classified as receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Both RLKs and RLPs contain an extracellular domain for ligand binding and a transmembrane domain. The intracellular kinase domain is only present in RLKs (Zipfel, 2014; Holton *et al.*, 2015). Leucine-rich repeat receptor-like kinases (LRR-RLKs) comprise the largest group within the family of Receptor-like kinases (RLKs) (Shiu *et al.*, 2004). The best characterised PAMP/PRR signalling complex is the flg22, recognised by the LRR-RLK receptor flagellin sensing 2 (FLS2) in Arabidopsis (Gómez-Gómez & Boller, 2000). Another bacterial flagellin derived peptide, flgII-28, was shown to elicit the induction of reactive oxygen species (ROS) in tomato and *N. benthamiana* but not in Arabidopsis and bean, suggesting it is a MAMP only recognised by solanaceous species (Cai *et al.*, 2011). Recently

a different receptor from tomato, Flagellin sensing 3 (FLS3), was shown to recognise this region of flagellin (Hind *et al.*, 2016).

The *A. thaliana* receptor EFR was shown to be responsible for recognition of bacterial EF-Tu (Zipfel *et al.*, 2006), while another Arabidopsis LRR-RLP Responsiveness to Botrytis Polygalacturonase1 (RBPG1) recognises fungal PGs (Zhang *et al.*, 2014).

Brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1), also known as SERK3 from the somatic embryogenesis receptor kinases (SERKs) family (Hecht *et al.*, 2001), was initially described as a co-receptor involved in Brassinosteroid-Insensitive1 (BRI1)-mediated Brassinosteroid (BR) signalling pathway (Li *et al.*, 2002). BAK1 is also required for PTI responses and has been shown to be involved in FLS2 and EFR signalling (Chinchilla *et al.*, 2007).

BIR1 is a receptor-like kinase dependant on BAK1 and it is a negative regulator of defence responses (Gao *et al.*, 2009). In contrast, LRR-RLK suppressor of BIR1-1 (SOBIR1) is important for the induction of defence responses in the Arabidopsis bak1-interacting receptor-like kinase 1,1 (bir1-1) mutant plants (Gao *et al.*, 2009). Furthermore, SOBIR1 functions mostly as an adaptor of the RLP- type PRRs. For example, it was shown to be required for the Cf-2, Cf-4, and Ve1-mediated defence responses (Liebrand *et al.*, 2013).

When PRR/BAK1 complexes are formed following PAMP perception, they lead to the activation of downstream signalling pathways. These pathways involve the production of reactive oxygen species (ROS), papillae formation, an increase in cytosolic Ca^{2+}levels, the activation of mitogen-activated protein kinase (MAPK) cascade and the up-regulation of defence related genes (Cecchini *et al.*, 2009; Nicaise *et al.*, 2009; Boudsocq *et al.*, 2010; Segonzac *et al.*, 2011).

It has been reported that certain PRRs can be present or absent in different plant species. New findings show that the transfer of these receptors across plant species/families is a very promising biotechnological strategy to improve plant resistance (Wulff *et al.*, 2011). EF-Tu receptor, only found in Brassicaceae species, was shown to induce defence responses to efl18 in *N. benthamiana* and tomato (*Solanum lycopersicum*) which lack this receptor (Lacombe *et al.*, 2010). Also, resistance to *Xanthomonas axonopodis* pv. c*itri* was achieved by the transfer of the rice receptor kinase XA21 into citrus (*Citrus sinensis*) (Mendes *et al.*, 2010).

1.2.4 Plant defence related genes activation upon PAMP perception

Many different genes are transcriptionally inducible after PAMP perception. Examples of these genes are *WRKY7* and *WRKY8* that belong to a family of transcription factors (Eulgem *et al.*, 2000). It has been shown that silencing of the *WRKY8* transcription factor using virus induced gene silencing (VIGS) in *N. benthamiana* increased the pathogenicity of the potato pathogen *P. infestans*, suggesting an important role of WRKY8 in plant defence responses (Adachi *et al.*, 2015). The clustered serine-proline residues (SP cluster) within the N-terminal region of WRKY8 is phosphorylated by mitogen-activated protein kinases (MAPKs), the salicylic acid-induced protein kinase (SIPK) and the wounding-induced protein kinase (WIPK) (Ishihama *et al.*, 2011). WRKY7 is also phosphorylated by active MAPKs during plant defence responses (Adachi *et al.*, 2015). Both WRKY7 and WRKY8 are required for ROS activation by the Mitogen-activated Protein Kinase Kinase 2 (MEK2) cascade regulating defence gene expression downstream of this cascade. Moreover, they are involved in the expression of NADPH oxidase gene RBOHB induced by *P. infestans* elicitin INF1 and the interaction between RXLR cytoplasmic effector AVR3a from *P. infestans* with potato resistance protein R3a (Adachi *et al.*, 2015). In the same way, the fungal Avr9/Cf-9 Rapidly Elicited (*ACRE*) gene expression profiling showed that they were induced or elicited during Avr9/Cf-9-mediated defence response in tobacco cell cultures (Durrant, 2000). It has also been shown previously that *ACRE* genes are upregulated after treatment with flg22 from *P.*

syringae in *N. benthamiana* (Heese *et al.*, 2007). Similarly, NbACRE31 was also induced by flg22 from the potato mollicute pathogen *Candidatus Liberibacter solanacearum* after infiltration into *N. benthamiana* (Hao *et al.*, 2014). Similarly, it has been shown that the expression of the SA-regulated genes *PR1* and *PR2* increased when pathogenesis-related genes transcriptional activators (Pti4/5/6) were expressed in Arabidopsis and bound to the ethylene-responsive element GCC box of the gene promoter, playing a role in plant defence responses (Gu *et al.*, 2002).

1.2.5 Pathogen effectors

In the race between plant and pathogen effectors are thought to play crucial roles. Effectors are small secreted proteins, delivered by the pathogen inside the plant cell or the apoplast to manipulate their hosts and facilitate infection by, among other processes, suppressing PTI and effector-triggered immunity (ETI) (Kamoun, 2006; Göhre & Robatzek, 2008; Stergiopoulos & de Wit, 2009). Effectors can be classified as apoplastic or cytoplasmic, according to the targeting site in the host (Kamoun, 2006). Apoplastic effectors are secreted and fulfill their pathogenicity functions outside of the plant cells. The cysteine residues characteristic of these effectors are thought to form disulfide bridges which regulate their stability in the apoplast (Kamoun, 2006). Examples of apoplastic effectors include Avr2, Avr4, Avr9 from C. *fulvum* (Thomma *et al.*, 2005), necrosis-inducing proteins NIP1, NIP2 and NIP3 from *Rhynchosporium commune* (Kirsten *et al.*, 2012), and NLP proteins which are widely distributed across plant pathogens and may act as positive virulence factors, that accelerate disease and pathogen growth in host plants (Gijzen & Nürnberger, 2006). In contrast, cytoplasmic effectors target different components inside the host cell (Chaudhari *et al.*, 2014). The most well-known examples of cytoplasmic effectors are from the gram-negative plant pathogenic bacteria, translocated inside the host cell by type III secretion system (T3SS)

(Galan & Wolf-Watz, 2006) and the RXLR and Crinkler (CRN) families (Petre & Kamoun, 2014) widespread across oomycete pathogens (Schornack *et al.*, 2010).

1.2.5.1 Pathogen effectors helping to avoid or supress plant immunity

For its own advantage, pathogens have evolved strategies to overcome host resistance. There are special ways to turn a resistant plant cell into a susceptible one, indicated by the mechanism used by the pathogen to suppress or inhibit the plant defence responses, resulting in a susceptible host.

During infection, the pathogen secretes effector molecules to suppress PTI (Thomma *et al.*, 2011). Several effectors have been shown to play a crucial role in the avoidance or suppression of defence responses induced upon PAMP perception. For example, the *P. syringae* type III effectors HopF2 and HopAO1 expressed in transgenic *A. thaliana* plants were shown to suppress flg22-induced immune response (Guo *et al.*, 2009). In the same way type III effectors AvrPto and AvrPtoB also from *P. syringae*, suppress PTI responses by targeting RLKs including FLS2, CERK1, BAK1 (Göhre *et al.*, 2008; Xiang *et al.*, 2008; Gimenez-Ibanez *et al.*, 2009). Effector Avr3a from *P. infestans*, specifically AVR3aKI allelic form, suppresses the programmed cell death induced by extracellular protein elicitor INF1 through stabilization and inhibition of the ubiquitin E3-ligase CMPG1 (Bos *et al.*, 2006, 2010).

There are also some effectors which suppress the HR associated with ETI response. Clear examples of this HR-based programmed cell death (PCD) inhibition include AvrPtoB and AvrPto effectors (Pedley & Martin, 2003) from *P. syringae* pv. *tomato* DC3000 which are recognized by the Pto resistance protein from tomato (Salomon *et al.*, 2009). AvrPtoB is delivered into the plant cell to suppress PCD and the anti-PCD activity is found in its C-terminal region (Abramovitch *et al.*, 2006). In the same way, HopPtoD2 effector protein also from *P syringae* pv. *tomato* DC3000 (Jamir *et al.*, 2004) which possesses an N- terminal

domain similar to the one of the avirulence gene AvrPphD and a C-terminal domain similar to protein tyrosine phosphatases (PTPs), suppresses HR through a mechanism connected to its tyrosine phosphatase activity (Espinosa *et al.*, 2003).

Other manifestations of suppression of immune responses involve the avirulence proteins (Avr2, Avr4, Avr4E, and Avr9) from *C. fulvum* which are recognized by the tomato R proteins Cf-2, Cf-4, Cf-4E, and Cf-9 in the apoplast, respectively (Stergiopoulos & de Wit, 2009). One of the avirulence proteins, Avr2, suppresses the activities of the tomato cysteine protease Rcr3 that is required for the defence responses against *C. fulvum* (Rooney *et al.*, 2005).

1.2.6 The Zigzag model

The zigzag model was proposed to explain the role and evolution of the different molecular components involved in plant-pathogen interactions; it represents four phases of the plant immune system. The first active line of defence responses is the PAMPs or MAMPs recognition by PRRs resulting in PTI (Figure. 1.1). In the second phase, pathogens counterattack and the PTI responses are suppressed by effector proteins secreted by the pathogens resulting in effector-triggered susceptibility (ETS) (Figure. 1.1). In this evolutionary arms race, plants have evolved resistance (R) proteins which are able to recognise these effector molecules and induce ETI that is usually connected to HR responses. This is presented as phase 3 of the zigzag model (Figure. 1.1). In phase 4, pathogens try to avoid this recognition by losing or modifying the effector protein or by releasing new effector proteins capable of suppressing ETI (Jones & Dangl, 2006) (Figure 1.1).

Despite the fact that the zigzag model has been used by many plant pathologists through the years, and makes a clear differentiation of the components and the immune responses induced in each of the layers of defence, the line of distinction between PTI and ETI, even more between PAMPs and effectors, does not always fit its original definition (Thomma *et al.*,

2011). PAMPs were previously defined as conserved molecules within a group of microbe species, but Pep-13, a calcium-dependent transglutaminase, is conserved only in *Phytophthora* species (Brunner, 2002). Initially PAMPs, unlike effectors, were not expected to contribute to pathogenicity but it has been shown that the PAMP flagellin from *P. syringae* pv. *tabaci* plays a role in pathogenicity due to effect on motility (Taguchi *et al.*, 2006, 2010; Naito *et al.*, 2008). ETI is commonly associated with HR responses, but some PAMPs have been shown to induce the same type of response, for example, the PAMP Cellulose-Binding Elicitor Lectin (CBEL), a cell wall glycoprotein identified in *P. parasitica* var *nicotianae*, induces HR in tobacco (*Nicotiana tabacum*) and Arabidopsis (Khatib *et al.*, 2004). In contrast, some effector proteins have been shown to be widely conserved between species, as is the case with LysM effectors conserved in the fungal kingdom (De Jonge *et al.*, 2010).

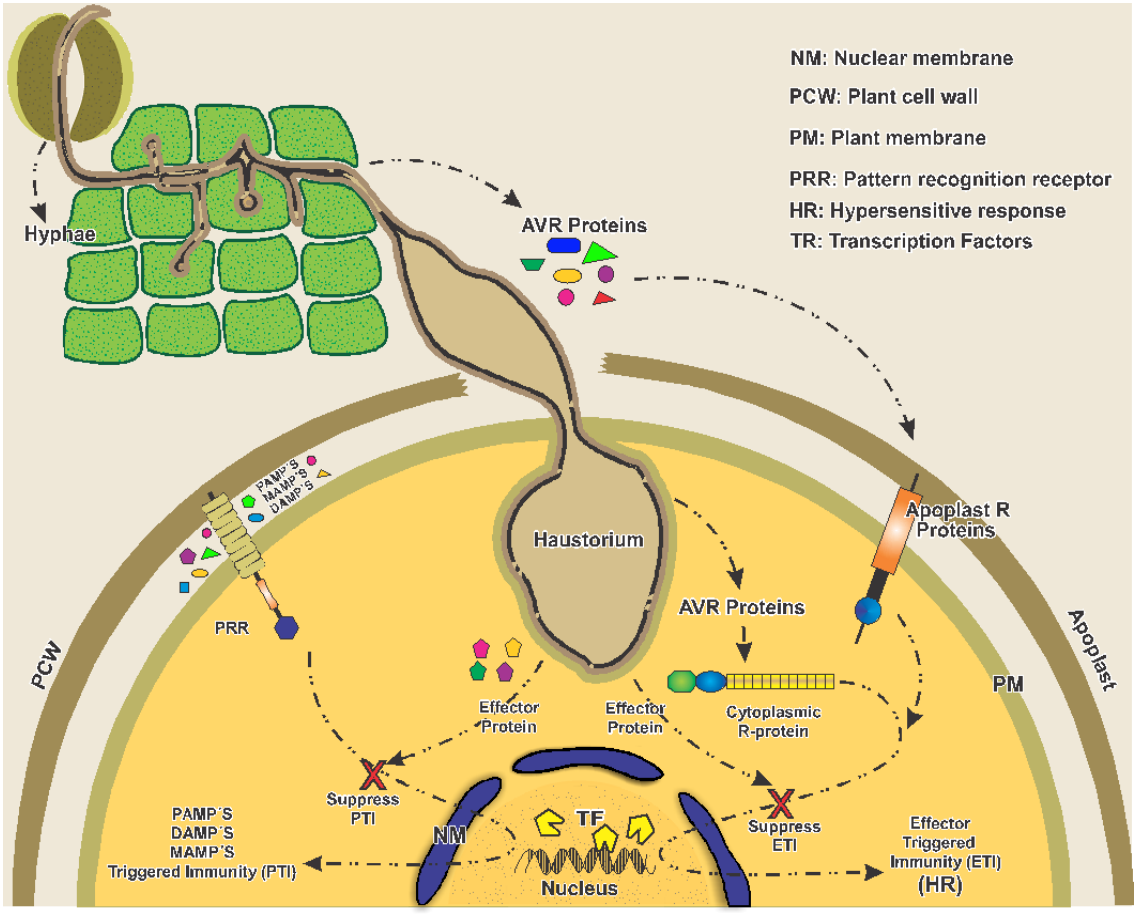


Figure 1.1 Plant-pathogen interaction system characteristic of biotrophic organisms (based on figure from Dodds & Rathjen, 2010). Pathogen- or microbe-associated molecular patterns (P/MAMPs) are recognized by pattern recognition receptors (PRRs) resulting in PAMP triggered immunity (PTI). Effectors are released from pathogens and interfere with PTI resulting in Effector triggered susceptibility (ETS). Effectors (avirulence proteins) are recognized by resistance (R) proteins resulting in effector triggered immunity (ETI) involving the activation of the plant defence response called Hypersensitive response (HR), corresponding to a cell death in the site of infection. ETI can also be suppressed by pathogen effectors. Cytoplasmic effectors are translocated into the cytoplasm where they can be recognised by NB-LRR proteins and apoplastic effectors can be recognized by plant cell surface receptors.

1.2.7 The role of SGT1 in plant immunity

The suppressor of G2 allele of Skp1 (SGT1) is a homologue of the yeast ubiquitin ligase-associated protein and is involved in several biological processes; importantly, it plays a special role in disease resistance through regulation of R proteins (Azevedo *et al.*, 2006). It was shown that silencing of SGT1 in a transgenic line of *N. benthamiana* expressing an HA-tagged Rx resistance protein from potato (*Solanum tuberosum*), reduced the levels of this resistance protein (Azevedo *et al.*, 2006). Thus, it was shown that SGT1 is essential for the HR triggered by the co-expression of R proteins Rx, Pto, Cf-4, and Cf-9 with their corresponding pathogen effector as ETI response, as well as for the HR induced by the *P. infestans* PAMP PiINF1 as PTI response when they were both suppressed in SGT1 silenced plants (Peart *et al.*, 2002). In addition, silencing of SGT1 in *N. benthamiana* compromised the non-host resistance against several bacterial pathogens, leading to infection (Peart *et al.*, 2002). The tetratricopeptide repeat (TPR) domain of SGT1 from Arabidopsis is required for resistance and auxin-mediated signalling response (Azevedo *et al.*, 2006).

1.2.8 The roles of jasmonic acid (JA) and salicylic acid (SA) in plant defence

JA and SA play important roles as mediators of disease resistance in plants (Journot-Catalino *et al.*, 2006). It has been shown that SA activates resistance responses against biotrophic pathogens; in contrast, JA activates defence responses against necrotrophs (Thomma *et al.*, 2001; Bari & Jones, 2009). Many different reports showed SA and JA signalling pathways performing a mutual antagonistic interaction (Glazebrook, 2005). For example, a clear

indication of JA suppression by SA signalling was shown in the transgenic NahG Arabidopsis plants, which are unable to accumulate SA (Spoel, 2003). The plants showed high levels of induction of the genes *LOX2*, *PDF1.2*, and *VSP* involved in the JA pathway against *P. syringae* pv *tomato* DC3000, in comparison to the moderate levels of expression of the same genes in Arabidopsis wild-type Columbia (Col-0) (Spoel, 2003). This cross-talk between SA and JA was shown to be mediated by the Nonexpressor of Pathogenesis-Related protein1 (NPR1) (Spoel, 2003). In the same way, the induction of SA signalling by the same bacterial pathogen suppressed JA signalling pathways, making Arabidopsis plants more susceptible to *Alternaria brassicicola* (Spoel *et al.*, 2007). However, compatibility between these two pathways has also been described in some cases. For example, the up-regulation of the SA marker gene *PR1* was not affected by the induction of JA-dependent induced systemic resistance (ISR) pathway in Arabidopsis (van Wees *et al.*, 2000). In the same way, microarray analysis showed that JA and SA-responsive genes are activated as a common defence response in rice against *Xanthomonas oryzae* pv. *oryzae* (Tamaoki *et al.*, 2013).

1.3 *Rhynchosporium commune*

Leaf scald caused by the fungus *Rhynchosporium commune* is one of the most destructive diseases of barley (*Hordeum vulgare*), principally in cool temperate climatic zones. Yield losses of 30-40% have been described as caused by this fungus (Shipton *et al.*, 1974). *R. commune* is a diverse pathogen with a high potential to evolve quickly due to host genetic changes, allowing the pathogen to evolve for its own benefit (Jackson & Webster, 1976; Zhang *et al.*, 1992; Xi *et al.*, 2002; Zhan *et al.*, 2008). *R. commune* has been found in all barley growing regions and was believed to originate from the Fertile Crescent (Middle East) which is the centre of origin of its host barley. Recent results suggest that northern Europe is the centre of origin of *R. commune* due to the highest genetic diversity present there (Zaffarano *et al.*, 2006).

1.3.1 Classification of *R. commune*

Relative to the type of reproduction, no sexual reproductive stage has been yet identified in *R. commune* populations (Arzanlou *et al.*, 2016). Previous studies aiming to characterize mating-type (MAT) idiomorphs of *R. commune* found that it was related to the heterothallic discomycetes *Pyrenopeziza brassicae* and *Oculimacula yallundae* (Foster & Fitt, 2003). The work done by Linde *et al*. (2003) provided some support for the suggestion of sexual reproduction for *R. commune*. Fungal isolates expressed mating type genes in equal frequency for most of the populations, which is consistent with sexual reproduction. In this same study both mating types, MAT1-1 and MAT1-2, were found in the same lesion area, giving the opportunity for the *R. commune* isolates belonging to one of the mating types to interact with the other one and reproduce sexually. In this case, syngamy was only going to be possible between gametes carrying the complementary mating types (Linde *et al.*, 2003).

Plant pathogens are also classified according to their lifestyle into biotrophs, hemibiotrophs and necrotrophs (Dou & Zhou, 2012). An example of a biotrophic organism is *Blumeria graminis* f.sp. *hordei*, causal agent of powdery mildew in barley (Dean *et al.*, 2012). It parasitizes living tissues to survive, and it is able to suppress HR to acquire all the nutrients it requires (Laluk & Mengiste, 2010). The development of specialized feeding structures termed haustoria is used for getting the nutrients from living cells (Perfect & Green, 2001).

B. cinerea is a typical example of a necrotrophic pathogen of a broad range of plant species, feeding of necrotic tissue (Williamson *et al.*, 2007). It is known that necrotrophic organisms promote the induction of the HR triggered by the host as resistance mechanism (Govrin & Levine, 2000). This plant defence response protects the host from biotrophic microbes that require living tissue to survive, but not from necrotrophic organisms which take advantage of necrotic tissue resulting in the spread of the disease (Dickman & de Figueiredo, 2013). An intermediate life style (known as hemibiotrophic) is where the pathogen penetrates and stays inside plant living tissues for the first period of life cycle and then it kills the tissue to take

nutrients (Erwin and Ribeiro, 1996). *Colletotrichum* species are typical examples of hemibiotrophic organisms (Münch *et al.*, 2008). *R. commune,* was initially classified as a necrotrophic fungus due to the necrotic lesions that it produces; however, currently it has been recognized as a hemibiotrophic pathogen specially for its long phase of growth between penetration and the appearance of disease symptoms (brown scald lesions) (Davis & Fitt, 1990). *R. commune* is able to sporulate and complete its life cycle without any expression of disease symptoms, an additional reason why it should be classified as biotroph in the first part of the infection process (Davis & Fitt, 1990; Thirugnanasambandam *et al.*, 2011). Nevertheless, haustoria, which play an important role for biotrophic and hemi-biotrophic fungi, have not been identified in *R. commune* (Oliver & Ipcho, 2004). In the necrotic stage, no evidence has been found of *R. commune* taking advantage of dead tissue or plant cell wall degradation (collapse of epidermal and mesophyll cells) to survive (Avrova & Knogge, 2012). To release nutrients from host cells, the fungus is believed to secrete compounds which have been shown to stimulate the plant plasma membrane-localized H+-ATPase, which may promote the essential membrane transport processes (Wevelsiep *et al.*, 1993). The definition of hemibiotroph is also assigned to other pathogens, for example, some *Colletotrichum* species (*C. lindemuthianum* and *C. destructivum*) (Mendgen & Hahn, 2002), *Magnaporthe oryzae* (Couch & Kohn, 2002) and *Zymoseptoria tritici* (Yang *et al.*, 2013) which have an initial asymptomatic phase and necrotic phase in the later stage of infection.

1.3.1.1 Host plants of *R. commune*

The first description made of genus *Rhynchosporium* was in rye around 1897 (Oudemans, 1897). The Genus *Rhynchosporium* was first described as two species: *R. secalis* and *R. orthosporium* (Zaffarano *et al.*, 2011). *R. orthosporium* is different from *R. secalis* due to the absence of beaked shaped form of conidia. *R. secalis* has been recognized as the pathogen infecting barley, rye, triticale and other grasses. Sequencing studies carried out by Zaffarano *et al*. (2011) helped to elucidate the specific host specialization of *Rhynchosporium*, splitting

it into 3 different species: *R. secalis* infecting triticle and rye; *R. commune* infecting barley, *B. diandrus* and other *Hordeum* spp.; and *R. agropyri* which infects *Agropyron* spp.

1.3.2 *R. commune* infection biology and epidemiology

The leaf symptoms of scald disease correspond to scald lesions of grey colour in early infection and dark brown margin with pale brown in the middle to later stages (Avrova & Knogge, 2012) (Figure 1.2a). During infection, *R. commune* conidia germinate on leaf surfaces which is followed by the penetration of the leaf cuticle using a germ tube. Direct penetration is carried out above anticlinal cell walls (Wevelsiep *et al.*, 1991), and then hyphal growth is restricted to the epidermis (Figure 1.2b). There is no penetration into epidermal or mesophyll cells (Jones & Ayres, 1974). At this stage no symptoms can be detected. This stage can be defined as the biotrophic phase in fungal development due to absence of cell damage (Zhan *et al.*, 2008). Later on, the necrotrophic phase is activated when epidermal and mesophyll cells collapse (Jones & Ayres, 1974; Lehnackers & Knogge, 1990). There is a long phase of asymptomatic growth between penetration and appearance of disease symptoms, clearly demonstrating the reasons for *R. commune* to be considered a hemibiotrophic fungus (Oliver & Ipcho, 2004). At the end of the infection process, the new conidia develop on conidiophores in healthy looking and affected regions (Davis & Fitt, 1990) (Figure 1.3).

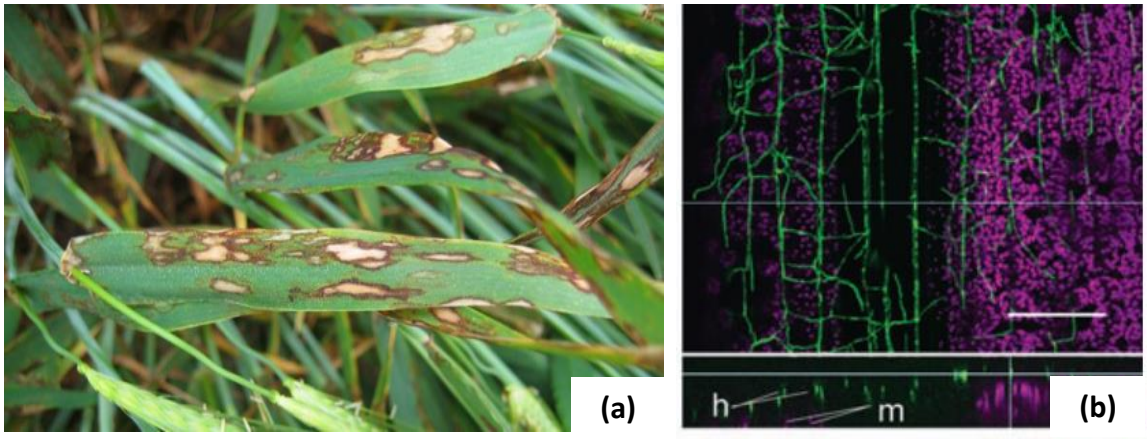


Figure 1.2 *Rhynchosporium commune* causes leaf scald disease in barley. (a) *R. commune* symptoms in barley leaf. (b) *R. commune* 214 GFP- fluorescing hyphae spreading throughout the sub-cuticular region of the epidermis (bar, 100 μm) (Thirugnanasambandam *et al.*, 2011; Walters *et al.*, 2012)

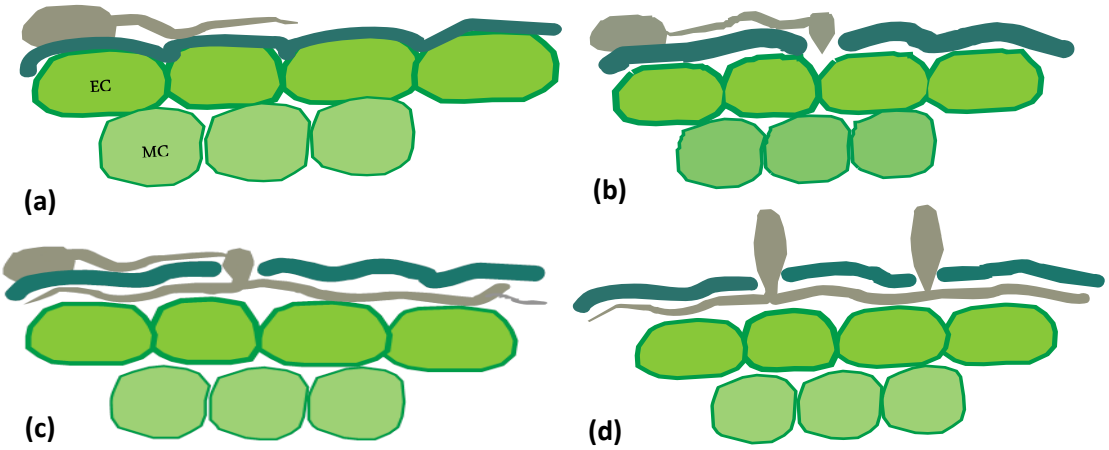


Figure 1.3 *Rhynchosporium commune* infection biology (based on figure from Zhan *et al.*, 2008). (a) Conidia germination on leaf surface. (b) Hyphae penetrating the cuticle directly above epidermal cells. (c) Fungal growth is confined to the subcuticular region of epidermis. (d) Formation of new conidia. EC: Epidermal cells. MC: Mesophyll cells.

R. commune inoculum remains on infected debris or seeds and is a primary source of propagation (Avrova & Knogge, 2012) (Figure 1.4). The secondary way of dispersion of infection is through rain splash from infected lower leaves upwards (Fitt *et al.*, 1988; Zhan *et al.*, 2008) (Figure 1.4).

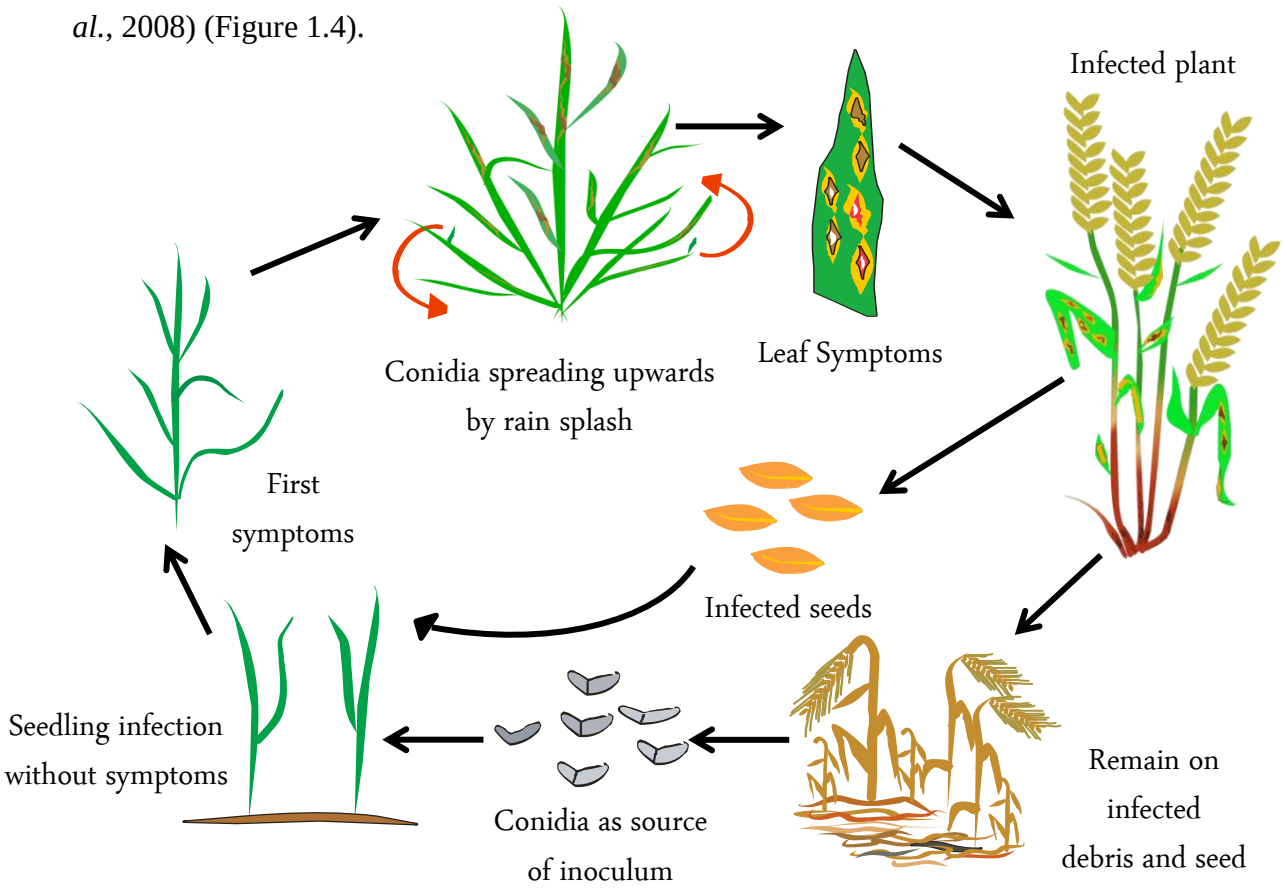


Figure 1.4 *Rhynchosporium commune* life cycle (based on figure from Avrova & Knogge, 2012)

1.3.3 Barley scald control

1.3.3.1 Agronomic practices and chemical control

Controlling *R. commune* in the field remains a challenge. Infected straw is one big source of inoculum for *R. commune* propagation (Skoropad, 1959). Other factors that promote the proliferation of *R. commune* in the field involve the reduction of tillage and continuous barley cultivation. In this case, crop rotation is highly recommended to reduce *R. commune* infection (Zhan *et al.*, 2008).

Fungicides have been often used to control the infection and propagation of *R. commune*, but fungicide resistance is another major potential threat to disease control (Avrova & Knogge, 2012). Fungicides like methyl benzimidazole carbamates (MBCs) and demethylation inhibitors (DMIs) were widely used for fungal control but resistant isolates have been identified (Taggart *et al.*, 1998, 1999). Despite this situation, DMIs are still used as important fungicides for effective control of *R. commune.* It is recommended the use of fungicides with different types of active ingredients (Walters *et al.*, 2012).

Despite the fact that management strategies have been implemented to control infections by *R. commune*, they are not completely effective due to the reasons previously mentioned. Therefore, the most sustainable plant disease control is through acquisition of genetic resistances (Zhan *et al.*, 2008; Avrova & Knogge, 2012). Plant resistance is the most suitable strategy to protect barley crops from *R. commune*, but for it to be completely efficient it must be durable. The genetic basis of such resistance could be only achieved by the understanding of the molecular basis underlying the plant-pathogen interaction which is the main focus of our study.

1.3.3.2 Race specific and race non-specific resistance to *R. commune*

Genetic resistance has been identified in host and non-host plants. Non-host resistance provides a broad-spectrum resistance against all different races and varieties of pathogen

(Kari & Griffiths, 1993; Karimi *et al.*, 2002). Host resistance is race-cultivar-specific and can be less durable (Mysore & Ryu, 2004). In the case of race specific resistance, some *R* genes from barley against *R. commune* have been identified (Shipton *et al.*, 1974); however the information provided in the literature is confusing and poorly understood. At the moment 16 major gene loci have been described as resistance genes against pathogen and a large number of quantitative trait loci (QTL) providing resistance to *R. secalis* (Zhan *et al.*, 2008). This last classification was based on the fact that some *R* genes previously described were just alleles of the same gene (Bjørnstad *et al.*, 2004); clear examples are the resistance QTL *q-4a, q-4b* and *q-4cd* co-localising with *R* genes *Rrs2, Rrs13* and *Rrs15,* and *q-7* locating with the *Rrs3* (Bjørnstad *et al.*, 2002).

Race specific resistance seems to be activated after the pathogen has penetrated host tissue; in contrast, race non-specific resistance can be activated before and after penetration (Zhan *et al.*, 2008). The first fungal avirulence gene identified in *R. commune* is the *AvrRrs1* (Rohe *et al.*, 1995). It encodes the secreted necrosis inducing protein NIP1 which induces plant defence responses in plants with the corresponding host resistance gene *Rrs1* (Hahn *et al.*, 1993).

Race non-specific or quantitative resistance is highly influenced by environment, through genotype by environment interactions (Kari & Griffiths, 1993) and is considered to be more durable than qualitative resistance (Walters *et al.*, 2012). It is thought to be regulated by multiple genes at quantitative resistance loci (Kirsten *et al.*, 2012). Several QTL have been found on six out of the seven barley chromosomes; none were found on chromosome 5H which also lacks *R* genes (Zhan *et al.*, 2008; Schweizer & Stein, 2011).

1.3.4 *R. commune* genome and transcriptome sequencing

Genome and transcriptome sequencing has been a powerful approach to understand the molecular mechanisms of the *R. commune*-barley interaction. For genome sequencing, three strains of *R. commune* and one strain of *R. secalis*, *R. agropyri*, *R. lolii* and *R. orthosporum* were sequenced using Roche 454 GS FLX and the Illumina GAIIx sequencing

platforms (Penselin *et al.*, 2016). Total sequence size was about 52-58 Mb. Gene numbers were estimated as 10254 to 13674 with about 50% of proteins with unknown function (Penselin *et al.*, 2016). Some of these sequences encode cysteine rich small secreted proteins.

Recent transcriptome sequencing of germinated conidia and barley epidermal strips infected with *R. commune* at 3 days post inoculation (dpi) was also carried out using Roche 454 GS FLX and the Illumina GAIIx sequencing platforms (Penselin *et al.*, 2016). For transcriptome sequencing, mRNA was isolated from germinated conidia and reverse transcribed into complementary DNA (cDNA), which was sequenced by 454 sequencing. For sequencing of the barley- *R. commune* interaction transcriptome, mRNA from the barley epidermal strips at 3 dpi with *R. commune* was sequenced by 76-bp single-end sequencing. The total sequence size was about 55 Mb. Data was assembled into 2,734 scaffolds, identifying 13,074 genes coding for proteins. Some of them have been used for further studies aiming for their characterization; three of them are referred to in this thesis as *RcCDI1*, *Rc2* and *Rsu3_07158*.

1.3.5 *R. commune* effectors

The interaction between *R. commune* and its host (barley) at the molecular level is still poorly understood. The first *R. commune* effectors identified were 3 NIP proteins purified from fungal culture filtrate (Wevelsiep *et al.*, 1991). Mature NIP1, NIP2, NIP3 are cysteine rich and contain 60, 93 and 98 amino acids, respectively (Kirsten *et al.*, 2012). These proteins are toxic to barley leaves and some dicotyledonous plants (Rohe *et al.*, 1995) causing necrosis resembling the disease symptoms (Wevelsiep *et al.*, 1991). NIP1 and NIP3 stimulate the plant plasma membrane H+-ATPase (Wevelsiep *et al.*, 1993), while no activity has been detected for NIP2. In contrast to NIP1, NIP3 was found in almost all *R. commune* isolates tested (Schürch *et al.*, 2004), therefore, at least one of the H+-ATPase stimulators, NIP1 or NIP3, appears to be present in a given *R. commune* isolate. While *NIP1* and *NIP3* occur as single

genes, *NIP2* families of 7-10 members are present in the genomes of closely related *R. commune, R. secalis and R. agropyri* (Penselin *et al.*, 2016).

NIP1 transcripts were detected in spores before fungal infection; in contrast, *NIP3* seems to be synthetized after leaf cuticle penetration by *R. commune* (Kirsten *et al.*, 2012). All 3 NIPs are upregulated early during barley infection, corresponding to the biotrophic phase of fungal development (Kirsten *et al.*, 2012).

In addition to the necrosis-inducing activity of NIP1, this protein was found to play a role in barley resistance against *R. commune.* As previously stated, it is the product of the avirulence gene *AvrRrs1* identified in *R. commune,* and it induces plant defence responses only in barley cultivars carrying the resistance gene *Rrs1* (Rohe *et al.*, 1995).

The interactions between Avr and R proteins are usually characterized by the induction of HR, but no HR is triggered upon recognition of NIP1 in barley. In addition, there is production of mRNAs encoding peroxidase and pathogenesis-related (PR) proteins in two barley resistant cultivars (Turk and Atlas 46) carrying *Rrs1* (Hahn *et al.*, 1993). It has also been shown that *R. commune* is able to overcome Rrs1 resistance with the deletion of *NIP1* (it is absent in 45% of isolates worldwide), or by alteration of NIP1 amino acid sequence (Schürch *et al.*, 2004). Comparison of NIP1 amino acid sequences from 200 isolates of *R. commune* led to the identification of 16 NIP1 variants, of which four (called types I-IV) have been widely studied. Different to variant types I and II, variants III and IV are not active in barley plants carrying resistance gene *Rrs1,* which is due to a single amino acid difference between them (Fiegen & Knogge, 2002).

More recently comparison of genome sequences of different *Rhynchosporium* species led to identification of 6 candidate effectors specific to *R. commune* (Penselin *et al.*, 2016). Unexpectedly, individual deletion of genes coding for these effectors yielded mutants that grew faster on susceptible barley cultivar Ingrid than the wild type strain UK7 (Penselin *et al.*, 2016).

1.3.6 Genetic transformation of filamentous fungi and gene deletion

Many transformation procedures have been developed for filamentous fungi (Galagan *et al.*, 2003). Successful transformants have been obtained for the fungus *A. nidulans* making use of protoplast transformation (Koukaki *et al.*, 2003). In the same way, the transformation of the protoplasts of *R. commune* strain UK7 was used to obtain deletion mutants for *NIP1* and *NIP3* (Rohe *et al.*, 1996). Using electroporation system, successful transformation have been performed for germinated conidia from filamentous fungi *N. crassa*, *Penicillium urticae, Leptosphaeria maculans, A. oryzae*, *Fusarium solani, A. nidulans* and *Trichoderma harzianum* (Richey, 1989; Goldman *et al.*, 1990). The biolistic transformation method has been used for *T. harzianum, Gliocladium* and *A. nidulans* (Chakraborty & Kapoor, 1990; Chakraborty *et al.*, 1991; Lorito *et al.*, 1993; Barcellos *et al.*, 1998). Finally, *A. tumefaciens* has been widely used as a transformation method for different plant and fungal species, including *A. awamori, A. niger* and *N. crassa* (de Groot *et al.*, 1998).

There are many methods to elucidate gene functions, including mutagenesis, gene replacement (knockout) or disruption by homologous recombination (Bhadauria *et al.*, 2009). These methodologies have been useful for characterising important genes involved in microbial pathogenicity after sequencing-derived gene discovery (Bhadauria *et al.*, 2009). In eukaryotes, two recombination pathways have been identified for DNA repair. In the first one, homologous recombination allows the repair of Double-Strand DNA Breaks (DSBs) using homologous templates. In the second, DNA breaks can also be repaired by Non-Homologous End Joining (NHEJ) which does not use homology to repair (the DNA is ligated directly) (Takata *et al.*, 1998). DSBs are repaired by both mechanisms but NHEJ is the most common mechanism in eukaryotic organisms (Hanin & Paszkowski, 2003).

Many filamentous fungi possess a very low rate of homologous recombination which makes the knockout procedure a challenging approach. A well-known example is the fungus *Neurospora crassa* which has very low levels of homologous recombination (10%) (Paietta &

Marzluf, 1985). In fungi, the frequency of homologous recombination has been increased with the deletion of *Ku70* or *Ku80* genes, as Ku70 and Ku80 heterodimers have been shown to affect the *NHEJ* pathway (Weld *et al.*, 2006). For example, disruption of the genes *mus-51* and *mus-52* (homologues of Ku70 and Ku80 respectively) involved in the NHEJ pathway in *N. crassa* increased the rate of homologous recombination to 100%, facilitating the production of knockouts for several *N. crassa* genes (Ninomiya *et al.*, 2004). In the same way, the use of *nkuA* deletion strain of *Aspergillus nidulans* provided an important tool to knock out many genes of interest in this fungus (Nayak, 2006). Moreover, recently successful gene editing has been achieved for filamentous fungus *N. crassa* and *Trichoderma reesei* using CRISPR/Cas9 technology (Liu *et al.*, 2015; Matsu-ura *et al.*, 2015).

R. commune protoplasts were first transformed with the genes conferring resistance to antibiotics hygromycin-B and phleomycin using polyethylene glycol (PEG)/calcium chloride ($CaCl_2$) treatment (Rohe *et al.*, 1996). *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been widely used. Thirugnanasambandam et al., (2011) showed successful transformation of *R. commune* with constructs for expression of the green fluorescent protein (GFP) and DsRed fluorescent protein to evaluate and track fungal growth in resistant compared to the susceptible barley cultivar. There was clear evidence of the difference in growth of *R. commune* in both cultivars at 3 days post inoculation (dpi). Even when the fungus was able to grow in the resistant cultivar Atlas 46 carrying resistance locus *Rrs1* in addition to *Rrs2* (Dyck & Schaller, 1961), the fungal growth was less extensive than in susceptible cultivar Atlas, carrying just resistance locus *Rrs2* (Dyck & Schaller, 1961; Lehnackers & Knogge, 1990). ATMT was also used by Kirsten, Siersleben, & Knogge (2011), but in this case *R. commune* transformed with eGFP gene was subjected to different antibiotics and a herbicide to test its sensitivity and the inhibitory effect of these compounds on fungal growth. Using protoplast transformation of wild type *R. commune* strain UK7, deletion mutants were achieved for the previously described *NIP1* and *NIP3* genes (Rohe *et*

al., 1996). *R. commune* conidia were transformed by ATMT to obtain *NIP2* deletion mutants (Kirsten *et al.*, 2012).

1.4. Scope of the thesis

R. commune is the pathogen responsible for one of the most devastating diseases of barley known as barley leaf scald (Zhan *et al.*, 2012). Some control management strategies have been used against the disease but they have not been completely effective due to the high genetic diversity of *R. commune* populations that can change rapidly overcoming barley resistance and fungicide control. Many different aspects of *R. commune* infection of barley are still unknown, especially the pathogenicity factors, including effectors. Some secreted effector proteins can play a role in suppressing the host immune system. Three NIP proteins have been identified in *R. commune* culture filtrate and characterised as pathogenicity factors of *R. commune* (Wevelsiep *et al.*, 1991; Kirsten *et al.*, 2012).

It is clear that the establishment of sustainable strategies for control against *Rhynchosporium* are directly linked to the increase in knowledge of the biology of the pathogen, the pathogen genes upregulated during the infection process, and the recognition of these pathogenicity factors by the host plant. Establishing functional genomic tools to discover pathogenicity/virulence determinants in *R. commune* would be hugely beneficial for the efforts to overcome scald disease. Such determinants could serve as good targets for the development of durable resistance.

Sequencing of the *R. commune* genome and RNA isolated from germinated conidia and an early stage of barley colonisation with *R. commune* revealed abundant transcripts coding for small secreted proteins. The analysis of these proteins can provide a valuable resource for identification of pathogenicity factors and it became the basis of this research project. The research described in this thesis aimed at the characterisation of three *R. commune* genes up-

regulated early during infection and coding for secreted proteins which we called RcCDI1, Rc2 and Rsu3_07158.

One of these candidate effectors, called RcCDI1, has been shown to cause cell death in *N. benthamiana* and was subsequently characterised as a novel PAMP from ascomycete fungi. It has been shown to trigger cell death in Solanaceous species, but not in monocots. Transient expression of truncated versions of RcCDI1 protein in *N. benthamiana* indicated that the co-expression of the two most conserved regions of the protein is essential for the recognition that triggers plant cell death. Identification of the Ascomycete PAMP RcCDI1, recognized by solanaceous species but not monocots is an important step toward identifying new resistance mechanisms that may be transferred between plant families. The ultimate goal would be to engineer globally important cereal crop plants wheat, rice and barley with durable resistance against Ascomycetes while avoiding adverse effects on plant growth and development.

Another *R. commune* candidate effector *Rc2* upregulated and highly abundant during early stages of barley colonisation was shown to code for a protein under Darwinian or positive selection. Unfortunately despite the presence of 2 alleles in sequenced *R. commune* strains no correlation was found between the virulence of sequenced *R. commune* strains tested in barley landrace SLB 10-009 and susceptible cultivar Optic, with amino acid changes in this protein sequence.

R. commune candidate effector *Rsu3_07158*, also upregulated early during infection, was shown to encode a putative hydrophobic surface binding protein A (HsbA). Attempts to obtain gene knockout for *Rsu3_07158* and *Rc2* to elucidate gene function by homologous recombination system were not successful.

In summary, this work gives us new insights into the potential role of molecules released by *R. commune* during its interaction with barley.

1.5 Aim and Objectives

The overall aim of this study is to elucidate the roles of *R. commune* candidate effector proteins in fungal pathogenesis by:

- Bioinformatics analyses of *R. commune* candidate effectors.
- Production of candidate proteins using *Pichia pastoris* and *Agrobacterium tumefaciens*-mediated transient expression systems.
- Gene expression profiling following protein infiltration.
- Targeted gene disruption via homologous recombination.
- Detached leaf assays to evaluate virulence.

The overall goal of this project is to understand the *R. commune*-barley interaction by the analysis of the molecules and mechanisms involved during infection to improve control strategies against this devastating pathogen.

Chapter 2. Materials and methods

2.1 Microbial cultures

R. commune strains L2A, L77, and AU2 from the culture collection at the James Hutton Institute were grown on CZV8CM agar medium (Newton, 1989) at 17^{o}C in the dark. Cultures were maintained by spreading spores from the surface of sporulating piece of mycelial mat to fresh CZV8CM agar plates every 2 weeks. Fungal conidia were harvested from approximately fourteen-day-old cultures by scraping the mycelial mat with a spatula following the addition of 5 ml of sterile distilled water (SDW). The conidial suspension was filtered through a funnel with glass wool and centrifuged for 3 min at 1600 x g. Following pellet resuspension in 1 ml of SDW spore concentration was checked using a haemocytometer. The inoculum concentration was adjusted to 1x10^{6} spores/ml.

A. tumefaciens and *Escherichia coli*, used in cloning experiments, were cultured at 28°C and 37°C respectively in Luria-Bertani (LB) medium using appropriate antibiotics. All bacterial DNA transformations were conducted by electroporation using standard protocols (Sambrook & Russell, 2001).

2.2 Infection time course and virulence testing

2.2.1 Barley growth

Plants of the barley (*Hordeum vulgare* L.) landrace SLB 10-009 and the highly susceptible cv Optic were grown under glasshouse conditions at 19^{o}C and 16 h day length.

2.2.2 Inoculation and trypan blue staining of *R. commune in planta*

Ten-day-old plants of barley cv Optic were spray-inoculated with suspensions of *R. commune* strain L2A conidia (10^{6} spores/ml, 0.1% Tween 20) and kept in plastic boxes at 100% humidity for 72 h with the first 24 h in the dark. After 72 h the inoculated plants were kept at 80% relative humidity. Leaf samples were taken before inoculation, and at 1, 2, 3, 4, 6, 8, 10

and 13 days post inoculation (dpi). To allow for variation in infection, leaf sections from five plants were collected for each time point, frozen in liquid nitrogen and stored at -80^{o}C prior to RNA extraction. Additional inoculated plants kept for 22 dpi showed high levels of infection. Uninoculated plants remained symptomless. Leaf samples were also taken at 3, 4, 6, 8, 10 and 13 dpi for trypan blue staining (Koch & Slusarenko, 1990) and light microscopy, to confirm the stages of infection as conidia germination and penetration (1-3 dpi), the biotrophic interaction with internal hyphae spreading under the cuticle (3-8 dpi), and a transition phase between biotrophy and necrotrophy (10-13 dpi).

2.2.3 Detached leaf assay

Leaves from ten day old barley plants were used for detached leaf assays performed as described in Newton *et al.* (2001). Briefly, 3-4 cm sections of leaf from each cultivar were cut and placed into rectangular polystyrene boxes (79 x 47 x 22 mm) (Stewart Solutions) filled with approximately 20 ml of 0.5% water agar supplemented with benzimidazole (120 µg/ml) (Sigma). Before inoculation the surface of the leaf was brushed to remove the wax layer. 10 µl of the suspension of *R. commune* conidia were then pipetted onto the surface of each leaf section. The boxes were placed in a light incubator (Leec, model LT1201) set at 17°C and 16 h day length. After 7-10 days the inoculum droplet disappears, and lesions start to form. The lesions were measured daily. The length of the lesions was measured as long as the leaves stayed green and free from contamination.

2.3 RNA isolation and cDNA synthesis

Total RNA was extracted from conidia and conidia germinated in SDW for 24 h using a Qiagen RNeasy Plant mini kit, following the manufacturer's protocol. RNA integrity was tested by gel electrophoresis. RNA yield was measured using a NanoDrop Micro Photometer (NanoDrop Technologies Inc.). Prior to cDNA synthesis, RNA samples were DNaseI treated using the Ambion Turbo DNA-free kit, following the manufacturer's instructions. mRNA was

extracted from inoculated leaf samples using Dynabeads Oligo $(dT)_{25}$ (Invitrogen Dynal AS). First strand cDNA for quantitative RT-PCR (qRT-PCR) was synthesised from 10-15 µg of total RNA or 150 ng mRNA by oligo dT priming using the SuperScript III Reverse Transcriptase (Invitrogen), following the manufacturer's protocol.

2.4 Bioinformatics

Signal peptide and position of cleavage site predictions were performed using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) using default settings. RcCDI1, Rc2 and Rsu3_07158 amino acid sequences were used for BLASTP searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with default settings to identify homologous proteins in other fungi. The ClustalW programme with default settings in the BioEdit software package was used to align DNA and protein sequences. Phylogenetic dendrogram of the mature protein sequences was constructed using MEGA6 (www.megasoftware.net) with maximum likelihood (Tamura *et al.*, 2013). O-(alpha)-GlcNAc and N-linked glycosylation sites were predicted using CBS Prediction Servers using default settings (http://www.cbs.dtu.dk/services/).

2.5 qRT-PCR assays

SYBR green qRT-PCR assays for gene expression analysis were carried out as described in Avrova *et al.* (2003). *R. commune actin, c-4 methyl sterol oxidase* and *delta-9 fatty acid desaturase, N. benthamiana* domain *tubulin* and *elongation factor 1α* and barley *elongation factor 1α* were used as constitutively expressed endogenous control genes. Relative expression of *RcCDI1* was normalized against expression levels in conidia; the expression of *N. benthamiana* PTI marker genes was normalized against pre-infiltration levels as described in Grenville-Briggs *et al.* (2008) and the expression of barley SA and JA marker genes was normalized against water infiltrated leaves. Assays were performed using cDNA from three independent infection time courses, except for the relative expression of *Rc2* and *Rsu3_07158*

where cDNA from just one infection time course was used. The expression of barley SA and JA marker genes was performed using data from one experiment. Primer sequences used in these assays are provided in Table 2.1.

Table 2.1 Oligonucleotide primers list.

Primer name	Primer sequence 5'-3'	Purpose
RcCDI1TMF	CGCTGGCTACTTGGACAACA	qRT-PCR for expression profiling of *RcCDI1*
RcCDI1TMR	CACGCATACCAGCGATAGTAAGC	
RcActTMF	GCGAGGACGACCAACGAT	qRT-PCR for expression profiling of *actin*
RcActTMR	AATGTGTAAGGCCGGTTTCG	
Rcc-4TMF	GGTGGGATTACATGATGGACACT	qRT-PCR for expression profiling of *c-4 methyl sterol oxidase*
Rcc-4TMR	CTGGACCTTCTTTGCCTTCTTC	
RcacylTMF	CGCTGGTGTTGTCCACGAT	qRT-PCR for expression profiling of *delta-9 fatty acid desaturase*
RcacylTMR	CTTGCCAATACCGGAGGTGAT	
P35S-FOR	AAGGAAGTTCATTTCATTTGGAGAGGA	Colony PCR and sequencing for PK7RWG2 plasmid after LR reaction
T35S-REV	CAACACATGAGCGAAACCCTATAAGAA	
SP-RcCDI1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGCATCTTTCTATCCTCACCAGC	Amplification of *SP-RcCDI1* from genomic DNA
SP-RcCDI1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCGATGAACACCCGCTTGAC	Amplification of *SP-RcCDI1* and *RcCDI1–SP* from genomic DNA
RcCDI1-SPF	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGCAATATTACAATGTTACGTCTAAGCCATTCC	Amplification of *RcCDI1–SP* from genomic DNA
SP-SsCDI1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGCGTACCTCATTCATCGCCACG	Amplification of *SP-SsCDI1* from genomic DNA
SP-SsCDI1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCCTCTACAAAGACCCTCTTCAC	
SP-BcCDI1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGCGTACCTCATTTATCCTCACT	Amplification of *SP-BcCDI1* from genomic DNA
SP-BcCDI1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCGGTGAAAACTCTTTGAAC	

EPIC1gatewayF1	AAAGCAGGCTTCACCATGGGCGGATACTCGAAGAAG	Amplification of *SP-PiEpiC* from genomic DNA
EPIC1gatewayR1	GAAAGCTGGGTCCTACTTAACTGGGGTAATCGA	
M13F	GTAAAACGACGGCCAG	Colony PCR and sequencing for pDONR201after BP reaction
M13R	CAGGAAACAGCTATGAC	
NbTubulinF	ATCGCATCCGAAAGCTTGCAG	qRT-PCR endogenous control for *NbBAK1* and *NbSGT1* expression levels after VIGS treatment
NbTubulinR	ACATCAACATTCAGAGCTCCATC	
BAK1F	GAGGTGGGAGGAATGGCAAA	qRT-PCR for *NbBAK1* expression levels after VIGS treatment
BAK1R	TTGGCCCCGACAATTCATCT	
SGT1F	GAAGTGATGTCCACCAAAATTG	qRT-PCR for *NbSGT1* expression levels after VIGS treatment
SGT1R	CCCATTTCTTCAGCTCCATGCC	
NbSOBIR1TMF	CCAGCAAGTCACAGAAGGGA	qRT-PCR for *NbSOBIR1* expression levels after VIGS treatment
NbSOBIR1TMR	CCAACACCACACCAAAGCTG	
qRT_Pia5_F	CCTCCAAGTTTGAGCTCGGATAGT	qRT-PCR for *NbPIA5*
qRT_Pia5_R	CCAAGAAATTCTCCATGCACTCTGTC	
qRT_Acre31_F	AATTCGGCCATCGTGATCTTGGTC	qRT-PCR for *NbACRE31*
qRT_Acre31_R	GAGAAACTGGGATTGCCTGAAGGA	
qRT_NbWRKY7_F	CACAAGGGTACAAACAACACAG	qRT-PCR for *NbWRKY7*
qRT_NbWRKY7_R	GGTTGCATTTGGTTCATGTAAG	
qRT_NbWRKY8_F	AACAATGGTGCCAATAATGC	qRT-PCR for *NbWRKY8*
qRT_NbWRKY8_R	TGCATATCCTGAGAAACCATT	
qRT_Nb-ef1aF	TGGACACAGGGACTTCATCA	qRT-PCR endogenous control for *N. benthamiana*
qRT_Nb-ef1aR	CAAGGGTGAAAGCAAGCAAT	
Rc2F	GTATCTCTCGAGAAAAGAGAGGCTGAAGCTACAATGGATGTGATGCAAGCTTTG	*Rc2* amplification from *R. commune* genomic DNA for expression in *Pichia pastoris*
Rc2R	AGCTCCGGCACCAGCACCGGCACCAGCTCCGCAAGTAAGTAAATGGGTACCATTTGTG	
MGF1	GTATCTCTCGAGAAAAGAGAGGCTGAAGCTCAGATCGAGGACCTCGAGACATCG	*MoCDI1* amplification from genomic DNA for expression in *P. pastoris*
MGR1	GTGTCACGGCAGGCGTACCAGTTGTACATTGCATGCGACG	

MGF2	CAATGTACAACTGGTACGCCTGCC GTGACACGC	*MoCDI1* amplification from genomic DNA for expression in *P. pastoris*
MGR2	AGCTCCGGCACCAGCACCGGCACC AGCTCCCTCGAAAACCCGATGAAT AGTGACAG	
NEF1	GTATCTCTCGAGAAAAGAGAGGCT GAAGCTCAGACCTACACCCAAGAT GGCCC	*NcCDI1* amplification from genomic DNA for expression in *P. pastoris*
NER1	ATCCTCCGAAATTTGCGCAACCTT GGCCACCTTC	
NEF2	GTGGCCAAGGTTGCGCAAATTTCG GAGGATGACCATGC	*NcCDI1* amplification from genomic DNA for expression in *P. pastoris*
NER2	AGCTCCGGCACCAGCACCGGCACC AGCTCCGAACTGGCCATCGTTGCT TTGAG	
ZTF	GTATCTCTCGAGAAAAGAGAGGCT GAAGCTCAGACGACCCAGTCTGCA CCTTTC	*ZtCDI1* amplification from genomic DNA for expression in *P. pastoris*
ZTR	AGCTCCGGCACCAGCACCGGCACC AGCTCCATAGGCCGCAGAGTATCT CCTCAC	
RcCDI1F	GTATCTCTCGAGAAAAGAGAGGCT GAAGCTCAATATTACAATGTTACG TCTAAGCCATTCC	*RcCDI1* amplification from *R. commune* genomic DNA for expression in *P. pastoris*
RcCDI1R	AGCTCCGGCACCAGCACCGGCACC AGCTCCGATGAACACCCGCTTGAC ATCC	
PGR106F	TGTACTAAAGAAATCCCCATC	Colony PCR for PVX plasmid PGR106
PGR106R	ATCACAGTGTTGGCTTGC	
INF1F	GTATCTCTCGAGAAAAGAGAGGCT GAAGCTACCACGTGCACCACCTCG CAG	*INF1* amplification for expression in *P. pastoris*
INF1R	AGCTCCGGCACCAGCACCGGCACC AGCTCCTAGCGACGCACACGTAGA CGA	
PGAP F	GTCCCTATTTCAATCAATTGAA	P75 plasmid sequencing
AOXTT R	GCAAATGGCATTCTGACATC	
MCH F	CGAAGTTCATCACGCGCTCC	
PGAPUP F	GCCCCCTTGCAGCAATG	Colony PCR after *P. pastoris* transformation
ALPHA R	AGCTTCAGCCTCTCTTTTCTCGAG AGATAC	
2A-F	ACAGCTCTTAAACTTTGACCTACT TAAGTTAGCAGGTGACGTTGAGTC CAACCCAGGACCGGGCC	Cloning FMDV 2A into the pCa-γbLIC vector
2A-R	CGGTCCTGGGTTGGACTCAACGTC ACCTGCTAACTTAAGTAGGTCAAA GTTTAAGAGCTGTGGCC	
ilov.2A-F	CCAACCCAGGACCGTTGATGatagag aagaatttcgtca	Amplification of *iLov* for *in planta* expression from BSMV
ilov.myc-R	AACCACCACCACCGTTATAAATCT TCCTCACTTATTAATTTTTGTTCtaca	

	tgatcacttccatcgagctgcac	
Rs1_03470.2A-F1	CCAACCCAGGACCGTTGATGcatcttt ctatcctcacca	Amplification of *RcCDI1* for *in planta* expression from BSMV
Rs1_03470.myc-R1	AACCACCACCACCGTTATAAATCT TCCTCACTTATTAATTTTTGTTCgat gaacacccgcttgacat	
RsNip1.2A-F1	CCAACCCAGGACCGTTGATGatgaaat tcctcgtactgcctct	Amplification of *RcNip1* for *in planta* expression from BSMV
RsNip1.myc-R1	AACCACCACCACCGTTATAAATCT TCCTCACTTATTAATTTTTGTTCacat tggcggtatcccgtcg	
RcCDI1-P1	GGAAGGGCGATCGGTGCGGGCCG TTTAAAC AAAGCTGCTCCTGCCTACGG	Amplification of left flaking region for deletion cassette
RcCDI1-P2	TTGTGTCATGAATTAACAGTTAAC GAATACACGGAGGAGGAAGATGT TGG	
RcCDI1-P3	TTAGTGTCAAACAGTCAAACCAGT TCTACGAGACTCGGAGGAAGTGG GACG	Amplification of right flaking region for deletion cassette
RcCDI1-P4	TGGAATTGTGAGCGGATAACAAGT TTAAACTTCAATTACCTGGCGAGT GC	
Rc2-P1	GGAAGGGCGATCGGTGCGGGCCG TTTAAACACTCGATCTTCTCCCCAT GCG	Amplification of left flaking region for deletion cassette
Rc2-P2	TTGTGTCATGAATTAACAGTTAAC GAATACAGAAGCAAGCAAAGTCG TGGC	
Rc2-P3	TTAGTGTCAAACAGTCAAACCAGT TCTACG TTCCTCCAGCTGTTTCAAGC	Amplification of right flaking region for deletion cassette
Rc2-P4	TGGAATTGTGAGCGGATAACAAGT TTAAAC TCTGCTTCTCATGATGGTGC	
Rsu3_07158-P1	GGAAGGGCGATCGGTGCGGGCCG TTTAAAC CACTTGTGGAGGCATAAACC	Amplification of left flaking region for deletion cassette
Rsu3_07158-P2	TTGTGTCATGAATTAACAGTTAAC GAATACTCTTGTAGAGCACCCAGA CTC	
Rsu3_07158-P3	TTAGTGTCAAACAGTCAAACCAGT TCTACG AAGTCCTGCGGTTCATTTGC	Amplification of right flaking region for deletion cassette
Rsu3_07158-P4	TGGAATTGTGAGCGGATAACAAGT TTAAAC GGTTATTGAACGTGGTGGTC	

RcCDI1-WTFL	TCTTATGCTGGTGTCTTGGC	Amplification of left flaking of the wild type gene during genotyping PCR
RcCDI1-WTRL	AAGGAGCCATGTGACAAGACC	
RcCDI1-WTFR	TCAATGTTTCCTCCTCTTTGC	Amplification of right flaking of the wild type gene during genotyping PCR
RcCDI1-WTRR	TGGACAAAGACTCCTTGAGGC	
Rsu3_07158-WTFL	AAATCTTGAGTTCACCAGCCG	Amplification of left flaking of the wild type gene during genotyping PCR
Rsu3_07158-WTRL	TGTAGTGCCATTGACAGTCGG	
Rsu3_07158-WTFR	TCTTGCAGTCCTTAGCGCGG	Amplification of right flaking of the wild type gene during genotyping PCR
Rsu3_07158-WTRR	AATCTGCTGGGAGTTCGTGGG	
hphF	ATGAAAAAGCCTGAACTCACCG	Amplification of the full length hygromycin gene
hphR	CTATTCCTTTGCCCTCGGACG	
HPHR	GACGATTGCGTCGCATCGAC	Amplification of the deletion cassette as split marker strategy
XX fw	GGAAGGGCGATCGGTGCG	
YY rv	GTGTGGAATTGTGAGCGGATAACAAG	Amplification of the deletion cassette as split marker strategy
hph split fw	GGGCGAAGAATCTCGTGCTTTCAG	
CC fw	GTATTCGTTAACTGTTAATTCATGACAC	Sequencing of left flaking region plus split hph PCR product
CC rv	TTGTGTCATGAATTAACAGTTAACG	
DD fw	TTAGTGTCAAACAGTCAAACCAG	Sequencing of right flaking region plus split hph PCR product
DD rv	CGTAGAACTGGTTTGACTGTTTG	
SP-RcCDI1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGcgaatatggcggacgaca	Amplification of *SP-RcCDI1* from genomic DNA
BgCDI1F-SPF	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGgacgacaattcaaattctagtgttattc	Amplification of *BgCDI1–SP* from genomic DNA
SP-BgCDI1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCtggatcgataaactttcgagtc	Amplification of *SP-BgCDI1* and Bg*CDI1–SP* from genomic DNA
$RcCDI1_{(1-99)}F$	GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCATGCATCTTTCTAT	Amplification of $RcCDI1_{(1-99)}$ domain

	CCTCACCAGC	with native signal peptide
RcCDI1$_{(1-99)}$R	GGGGACCACTTTGTACAAGAAAG CTGGGTCGGCGTTGCCGCCACGAA GGAG	
RcCDI1$_{(1-88)}$F	GGGGACAAGTTTGTACAAAAAAG CAGGCTTCACCATGCATCTTTCTA TCCTCACCAGC	Amplification of RcCDI1$_{(1-88)}$ domain with native signal peptide
RcCDI1$_{(1-88)}$R	GGGGACCACTTT GTACAA GAAAGCTGGGTCGGCAGTCTGGCC GGGTAGGCCTTG	
RcCDI1$_{(1-59)}$F	GGGGACAAGTTTGTACAAAAAAG CA GGCTTCACCATG	Amplification of RcCDI1$_{(1-59)}$ domain with native signal peptide
RcCDI1$_{(1-59)}$R	GGGGACCACTTTGTACAAGAAAG CTGGGTCGGCACCCACACACAGTC CTTC	
RcCDI1$_{(44-59)}$F	CACCAGCTGCCTCGCTCTTGCGAC TGGTATCTCAGCTCTCTTCGCATGT CACGAAGGA	Amplification of RcCDI1$_{(44-59)}$ domain with native signal peptide
RcCDI1$_{(44-59)}$R	GGGGACCACTTTGTACAAGAAAG CTGGGTCGGCACCCACACACAGTC CTTCGAT	
RcCDI1$_{(44-59)}$SP	GGGGACAAGTTTGTACAAAAAAG CAGGCTTCACCATGCATCTTTCTA TCCTCACCAGC	
RcCDI1$_{(156-200)}$F	CACCAGCTGCCTCGCTCTTGCGAC TGGTATCTCAGCTGCTCAAGCTTA CTATCGGTGG	Amplification of RcCDI1$_{(156-200)}$ domain with native signal peptide
RcCDI1$_{(156-200)}$R	GGGGACCACTTTGTACAAGAAAG CTGGGTCGGCGATGAACACCCGCT TGACATCC	
RcCDI1$_{(156-200)}$SP	GGGGACAAGTTTGTACAAAAAAG CA GGCTTCACCATGCATCTTTCTATCC TCACCAGCTGCCTCGCTCTTG	
RcCDI1$_{(89-155)}$F	CACCAGCTGCCTCGCTCTTGCGAC TGGTATCTCAGCTGGTCTTGTCAC ATGGCTCCTT	Amplification of RcCDI1$_{(89-155)}$ domain with native signal peptide
RcCDI1$_{(89-155)}$R	GGGGACCACTTTGTACAAGAAAG CTGGGTCGGCCTTGTAGACTGGTG GCGAAAC	
RcCDI1$_{(89-155)}$SP	GGGGACAAGTTTGTACAAAAAAG CA GGCTTCACCATGCATCTTTCTATCC TCACCAGCTGCCTCGCTCTTG	
RcCDI1Δ^{16} F	GGGGACAAGTTTGTACAAAAAAG CAGGCTTCACCATG	Amplification of RcCDI1Δ^{16} deletion

		mutant with native signal peptide
RcCDI1Δ^{16} R	TCGTACCCACTGCGAAGAGACCTTTTCCGTTCA	
RcCDI1Δ^{16} F	TCTCTTCGCAGTGGGTACGAGTGGTCCATC	
RcCDI1Δ^{16} R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGCGATGAACACCCGCTTGAC	
HvAOS-F	CTCTTCACCGGCACCTACAT	qRT-PCR for *HvAOS*
HvAOS-R	ACCGTCTTCAACAGCTACGG	
HvPR1-F	AGCACGAAGCTGCAGGCGTA	qRT-PCR for *HvPR1*
HvPR1-R	TCTCGTCCACCCACAGCTTCAC	

2.6 Plasmid construction

Plasmids used in this study (Table 2.2) were constructed using standard molecular biology techniques (Sambrook and Russell, 2001). The full-length *P. infestans* protease inhibitor *EpiC1* (XP_002903480.1) and the *BcCDI1, SsCDI1, BgCDI1, RcCDI1* coding gene sequences with and without signal peptide were amplified from genomic DNA from *P. infestans*, *B. cinerea*, *Sclerotinia sclerotium*, *B. graminis* and *R. commune*, respectively, using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and the primers listed in Table 2.1. Following gel purification using the QIAquick Gel Extraction Kit (Qiagen) the PCR products were recombined into pDONR201 Gateway vector using BP clonase (Invitrogen).

The plasmids were transformed by electroporation into *E. coli* ElectroMAX DH10B competent cells (Invitrogen) and positive clones selected on LB containing 50 µg/ml of kanamycin (Sigma). Sequence-verified constructs were recombined into the destination vector pK7RWG2 (expressing C-terminal mRFP fusions) (Karimi *et al.*, 2005) with LR clonase (Invitrogen) and transformed into *E. coli* ElectroMAX DH10B competent cells (Invitrogen). Positive clones were selected on LB agar supplemented with 100 µg/ml spectinomycin (Sigma). Sequence-verified constructs were transformed by electroporation into *A.*

tumefaciens strain AGL1-pVirG-pSOUP and selected using 100 µg/ml spectinomycin (Sigma), 25 µg/ml rifampicin (Sigma) and 25 µg/ml chloramphenicol (Sigma) for transient expression in *N. benthamiana*.

Barley stripe mosaic virus (BSMV) binary vector system comprising of three T-DNA binary plasmids, pCaBS-α, pCaBS-β and pCa-γbLIC (Yuan *et al.*, 2011), was used in this study. The pCa-γbLIC used was modified to allow the expression of small heterologous proteins as C-terminal fusions to BSMV γb via the FMDV 2A peptide bridge. pCa-γbLIC linearized with *Apa*I (New England Biolabs) was ligated with the oligonucleotides 2A-F and 2A-R, carrying coding sequence of the *Foot-and-mouth disease virus* (FMDV) 2A peptide, that were pre-annealed creating the vector pCa-γb2A-LIC (BSMV:00), which retains one *ApaI* site immediately downstream of FMDV 2A. The *iLov* (Chapman *et al.*, 2008) coding sequence was amplified from the TMV:iLov plasmid kindly provided by John Christie (University of Glasgow, UK).

Full-length coding sequences of *RcNip1* (Rohe *et al.*, 1995) and *RcCDI1* were amplified from germinated conidia of *R. commune* strain 214 and L2A, respectively. The resulting fragments were cloned into pCa-γb2A-LIC as described in Lee *et al.* (2015) to generate BSMV:*iLov*, BSMV:*RcNip1* and BSMV:*RcCDI1*. All of the primers used for plasmid construction are documented in Table 2.1.

Constructs for *PiINF1*, *R3a* and *AVR3a*^{KI} (Gilroy *et al.*, 2011), *PexRD2* (King *et al.*, 2014), *Cf-4* and *Avr4* (Van der Hoorn *et al.*, 2000) were described previously. Constructs used for VIGS of *NbBAK1*, *NbSGT1* and *NbSOBIR1* were used in the pTRV2 vector as described previously (Bos *et al.*, 2006; Heese *et al.*, 2007; Kettles *et al.*, 2017).

Table 2.2 Plasmid constructs list.

Plasmid name	Vector	Purpose
SP-RcCDI1	pK7RWG2	Expression of RcCDI1with signal peptide
RcCDI1-SP	pK7RWG2	Expression of RcCDI1without signal peptide
BgCDI1-SP	pK7RWG2	Expression of BgCDI1without signal peptide
SP-SsCDI1	pK7RWG2	Expression of SsCDI1with signal peptide
SP-BcCDI1	pK7RWG2	Expression of BcCDI1with signal peptide
SP-BgCDI1	pK7RWG2	Expression of BgCDI1without signal peptide
V5 (EV)	P75	*Pichia pastoris* expression system
PiInf1-V5	P75	INF1 expression in *P. pastoris*
RcCDI1-V5	P75	RcCDI1expression in *P. pastoris*
NcCDI1-V5	P75	NcCDI1 expression in *P. pastoris*
ZtCDI1-V5	P75	ZtCDI1 expression in *P. pastoris*
MoCDI1-V5	P75	MoCDI1expression in Pichia pastoris
BSMV: *RcCDI1*	pCa-γb2A-LIC	BSMV-based *in planta* expression system
BSMV:*iLOV*	pCa-γb2A-LIC	BSMV-based *in planta* expression system
BSMV:*Nip1*	pCa-γb2A-LIC	BSMV-based *in planta* expression system
TRV:BAK1	pTRV2	virus-induced gene silencing (VIGS)
TRV:SGT1	pTRV2	virus-induced gene silencing (VIGS)
TRV:EV	pTRV2	virus-induced gene silencing (VIGS)
TRV:SOBIR1	pTRV2	virus-induced gene silencing (VIGS)
Cf4	pRH48	Cf4 expression in *N. benthamiana*
Avr4	pRH87	Avr4 expression in *N. benthamiana*
PexRD2	pK7WGF2	PexRD2 expression in *N. benthamiana*
$Avr3a^{KI}$	pGR106	$Avr3a^{KI}$ expression in *N. benthamiana*
R3a	pGRAB	R3a expression in *N. benthamiana*
INF1	pCB302-3	INF1 expression in *N. benthamiana*
$RcCDI1\Delta^{16}$	pK7RWG2	Expression of RcCDI1 deletion mutant in *N. benthamiana*
$RcCDI1_{(1-99)}$	pK7RWG2	Expression of RcCDI1 domain with native signal peptide in *N. benthamiana*
$RcCDI1_{(1-88)}$	pK7RWG2	
$RcCDI1_{(1-59)}$	pK7RWG2	
$RcCDI1_{(44-59)}$	pK7RWG2	
$RcCDI1_{(156-200)}$	pK7RWG2	
$RcCDI1_{(89-155)}$	pK7RWG2	

2.7 Expression and purification of recombinant RcCDI1 and Rc2 protein homologues

Open reading frames for *Rc2* and *RcCDI1* homologues from *R. commune*, *Z. tritici*, *M. oryzae* and *N. crassa* were amplified from genomic DNA, and *P. infestans PiINF1* was amplified from an *Agrobacterium tumefaciens* strain harbouring *PiINF1* (Gilroy *et al.*, 2011) using primers with vector-specific 5' 30-bp extensions listed in Table 2.1. The intron present in

MoCDI1 and *NcCDI1* sequences was removed by amplifying the exons as separate amplicons that were subsequently fused together using yeast recombination cloning (YRC) (Oldenburg, 1997). Purified PCR products were combined with the linearized vector and added to the competent cells of *Saccharomyces cerevisiae* strain FY834 from the Fungal Genetics Stock Center (FGSC).

The pGAPZα plasmid (Invitrogen) was modified to introduce the V5 epitope, the *S. cerevisiae URA3* gene and 2 micron origin to allow it to be used for YRC. The pGAPZa plasmid was isolated from *S. cerevisiae* and the functional integration cassette was recovered by PCR or transformation of *S. cerevisiae* plasmid DNA into *E. coli* ElectroMAX DH10B competent cells (Invitrogen). The constructs were confirmed by sequencing. Plasmid was linearized with *Pme*I (New England Biolabs) prior to electroporation of *P. pastoris* strain GS115 (Invitrogen). Transformants were screened by colony PCR using primers described in table 2.1. Positive clones were grown in yeast extract-peptone-dextrose (YPD) medium (Melford Laboratories Ltd), containing 100 μg/ml zeocin, at 30°C, and shaking at 200 rpm. After 2-4 days cultures were centrifuged at 2500 xg for 5 min to produce supernatant containing RcCDI1 homologues. The presence of RcCDI1 homologues in supernatant was confirmed via high-sensitivity staining with SYPRO Ruby Protein Gel staining (Invitrogen) and immunoblotting using anti-V5 antibody before plant infiltration.

P. pastoris culture supernatants (CS) containing RcCDI1 homologues from *Z. tritici* and *M. oryzae* were concentrated 20 fold using Vivaspin 10 kDa MWCO columns (GE Healthcare Life Sciences). At the same time, YPD was exchanged for HNT buffer (50 mM pH7.4 HEPES-KOH, 50 mM NaCl, 0.02% Tween-20) to avoid increased concentration of *P. pastoris* proteins and other culture media constituents in the samples.

For the purification of recombinant RcCDI1 and PiINF1 proteins from the CS, 50 ml of cell-free YPD containing the recombinant RcCDI1 protein or PiINF1 was incubated for 1 h with the Anti-V5 affinity gel (Biotool) and poured onto Zeba Spin column (Thermo Scientific

Pierce). Subsequently columns were washed 4 times with TBS. RcCDI1-V5 and PiINF1-V5 were eluted using 200 µg/ml V5 peptide in TBS (20 mM Tris, 150 mM NaCl, pH 7.3). Amicon ultracentrifugal polypropylene Ultracel membrane 10KMWCO (Millipore) was used to exchange TBS to HNT buffer and concentrate the RcCDI1 and PiINF1 proteins. As a control, CS from *P. pastoris* expressing only the V5 tag was processed in the same manner in parallel.

2.8 *Agrobacterium* and protein infiltration assays

Agrobacterium-mediated transient expression was performed using 4-6 week-old *N. benthamiana* plants (Bos *et al.*, 2010). To assay for *RcCDI1*-triggered cell death *A. tumefaciens* strains carrying BSMV: *RcCDI1*, or as controls BSMV:00, BSMV:*iLOV* or BSMV:*RcNip1*, were infiltrated in parallel. Leaves were scored and photographed at six days post infiltration (dpi). Each construct was used to infiltrate leaves on at least three plants and on at least two occasions.

A. tumefaciens strain AGL1-pVirG-pSOUP carrying different constructs was tested for the ability to trigger cell death in *N. benthamiana* leaves. All the constructs were used in the pK7RWG2 plasmid containing the following sequences: *RcCDI1* with (*spRcCDI1*) or without signal peptide (*RcCDI1*) from *R. commune*, the *CDI1* gene with the native signal peptides from *B. cinerea* (*spBcCDI1*) and *S. sclerotiorum* (*spSsCDI1*), *RcCDI1* with (*spBgCDI1*) or without signal peptide (*BgCDI1*) from *B. graminis*, the truncated versions of *CDI1* gene with the native signal peptide from *R. commune* (*spRcCDI1*$_{1\text{-}99}$, *spRcCDI1*$_{1\text{-}88}$, *spRcCDI1*$_{1\text{-}59}$, *spRcCDI1*$_{44\text{-}59}$, *spRcCDI1*$_{89\text{-}155}$, *spRcCDI1*$_{56\text{-}200}$) and the *CDI1* deletion mutant for the most conserved 16 amino acid domain of RCDI1 protein (*spRcCDI1*Δ^{16}). Plant cell death was recorded at eight dpi and was counted as positive if more than 50% of the infiltrated area showed cell death.

To assay suppression of RcCDI1-triggered cell death by *P. infestans* RXLR effectors PiAvr3a^{KI} and PexRD2, *A. tumefaciens* strain AGL1 cells carrying each effector gene (pGR106-AVR3a^{KI} and pK7WGF2-PexRD2) were combined with *A. tumefaciens* carrying pGR106-spRcCDI1 prior to leaf infiltration. *A. tumefaciens* cultures for each construct were mixed in a 1:1 ratio in induction buffer (10 mM MES, pH 5.6 and 10 mM $MgCl_2$ buffer) supplemented with 0.2 mM acetosyringone to reach a final OD_{600} of 0.5 (King *et al.*, 2014). Plant cell death was recorded at eight dpi and counted as positive as explained above. All cultures were incubated for at least 1 h before infiltration into *N. benthamiana* leaves. PK7RWG2-empty vector (pK7RWG2-EV) construct was used as a negative control. Each assay consisted of at least eight plants infiltrated on three leaves on at least three occasions.

In the same way, *PiAVR3a^{KI}* was combined with *PiINF1* (pCB302-3-INF1) or its cognate resistance protein *R3a* (pGRAB-R3a). Similarly *PexRD2* (OD_{600} of 0.3) was mixed with tomato *Cf4* (OD_{600} of 0.6) and *C. fulvum Avr4* (OD_{600} of 0.3) in a 1:1:1 ratio. All cultures were incubated for at least 1 h before infiltration into *N. benthamiana* leaves. *A. tumefaciens* cultures for delivery of *R3a*, *PiAvr3a^{KI}*, *PiINF1*, *RcCDI1*, *SsCDI1* and *PexRD2* were also infiltrated into *N. benthamiana* leaves individually, and pGR106-empty vector construct was used as a negative control. Plant cell death suppression was recorded at seven dpi. Each assay consisted of at least eight plants infiltrated on three leaves on at least three occasions.

To test the induction of cell death by recombinant proteins produced by *P. pastoris*, 100 pM to 1 µM solutions of purified RcCDI1-V5 protein or equivalent amount of CS from *P. pastoris* expressing only the V5 tag, processed for protein purification, were infiltrated into leaves of *N. benthamiana*, while 100 nM solution of the purified RcCDI1-V5 protein was infiltrated into leaves of potato (*S. tuberosum* L.) cv Desiree, bean (*Vicia faba* L.) cv Sutton Dwarf and spinach (*Spinacia oleracea* L.) cv Amazon. Leaves of *N. sylvestris* Speg. & Comes, tomato (*S. lycopersicum* L.*) cv Moneymaker*, Arabidopsis (*A. thaliana* (L.) Heynh.) wild type Columbia (Col-0), barley cv Optic, wheat (*Triticum aestivum* L.) cv Tybalt, rye

(*Secale cereale* L.) and maize (*Zea mays* subsp. *mays* L.) cv Golden Jubilee were infiltrated with *P. pastoris* CS containing RcCDI1-V5. The same plants excluding potato, Arabidopsis, bean and spinach were infiltrated with *P. pastoris* CS containing Rc2-V5 and CS from *P. pastoris* expressing only the V5 tag. *N. benthamiana* leaves were photographed at three dpi and the leaves of the other species at seven dpi. Each assay consisted of three to four plants inoculated on two or three leaves on at least two occasions.

2.9 VIGS of *NbBAK1, NbSOBIR1* and *NbSGT1* in *N. benthamiana*

N. benthamiana plants were grown as described previously (Bos *et al.*, 2010). VIGS experiments were conducted in containment glasshouse under licence GM250.03.1. *A. tumefaciens* cultures transformed with *Tobacco rattle virus* (TRV) RNA1 vector and TRV RNA2 constructs (TRV:*BAK1*, TRV:*SGT1* or TRV:EV control) were mixed in a 1:1 ratio to achieve a final OD_{600} of 0.4 for the RNA1 constructs and OD_{600} of 0.5 for the RNA2 constructs (King *et al.*, 2014). Culture mixture was infiltrated into the two largest leaves of three weeks-old *N. benthamiana* plants. At two weeks post infiltration with VIGS constructs, plants were infiltrated with *P. pastoris* CS containing either RcCDI1-V5 or PiINF1-V5 proteins. CS of *P. pastoris* strain expressing V5 peptide only was used as a control. Plant cell death was scored at six dpi and counted as positive as described above. Each assay consisted of at least eight plants inoculated on three leaves and on at least two occasions. The silencing efficiency of *NbBAK1*, *NbSGT1* or *NbSOBIR1* was validated using qRT-PCR analysis of cDNA from leaves of six individual silenced and control plants at two weeks after infiltration with VIGS constructs.

2.10 Immunoblotting

Proteins were extracted from *N. benthamiana* leaves ground in liquid nitrogen using GTEN extraction buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl) (Oh & Martin, 2011), 2% w/v PVPP, 5 mM DTT, 1X protease inhibitor cocktail (Sigma), 0.1% NP-

40 (Fisher Scientific) in a 1:1 m/v ratio. Samples were centrifuged at 16000 g for 5 min at 4°C and subsequently the supernatant was used for gel electrophoresis in 4-12 % Bis-Tris gel NuPAGE Novex (Invitrogen) in a Novex® Pre-Cast Gel chamber (Invitrogen) following the manufacturer's protocol.

Proteins were transferred onto a nitrocellulose membrane for 90 min at 200 mA using an XCell SureLock® Mini-Cell and XCell II™ Blot Module (Invitrogen) following the manufacturer's protocol. After washing twice with water, the membrane was incubated in PBS blocking buffer (Bio-Rad) for 1 h at room temperature (RT) to reduce nonspecific binding. The membrane was then incubated at RT with the anti-V5-HRP antibody (Invitrogen) or Rat monoclonal RFP antibody (Chromotech GMBH) in blocking buffer for 1 h and 12 h, respectively, washed twice for 5 min with PBS-Tween (0.5%), and incubated with Goat anti-Rat HRP antibody (Insight Biotechnology) in blocking buffer for 1 h at RT for mRFP detection. The membranes were washed as previously described but with an additional final wash with PBS. Proteins were detected using SuperSignal™ West Pico chemiluminescent substrate (Fisher Scientific) and visualized on CL-XPosure film (Thermo Scientific).

2.11 Generation of *R. commune* gene knockouts

2.11.1 Integration cassette

Upstream and downstream regions of *RcCDI1*, *Rc2* and *Rsu3_07158* genes were amplified from genomic DNA using primers with extensions specific to the vector PRS426 and the Hygromycin-B-Phosphotransferase (hph) resistance marker (Table 2.1). Purified PCR products were combined with linearized vector and resistance marker and added to competent cells of *S. cerevisiae* strain FY834. The pGAPZα plasmid (Invitrogen) was modified as described above (Figure 2.1).

The plasmid pGAPZa was isolated from *S. cerevisiae* and the functional integration cassette was recovered by PCR as split-marker. This strategy consists of a PCR amplification which creates two PCR products, each containing a part of the resistant marker gene (Hygromycin) fused to one of the flanking regions of the gene. These two molecular cassettes were then simultaneously used for transformation into *R. commune* germinated conidia using electroporation procedure. Only the transformants in which the two overlapping fragments have successfully recombined will grow in selective medium. In the transformation procedure, to generate a gene knockout three homologous recombination events have to occur, one within each flanking region and one in the resistance marker gene. This should lead to the replacement of the gene of interest with the functional marker gene (Figure 2.1).

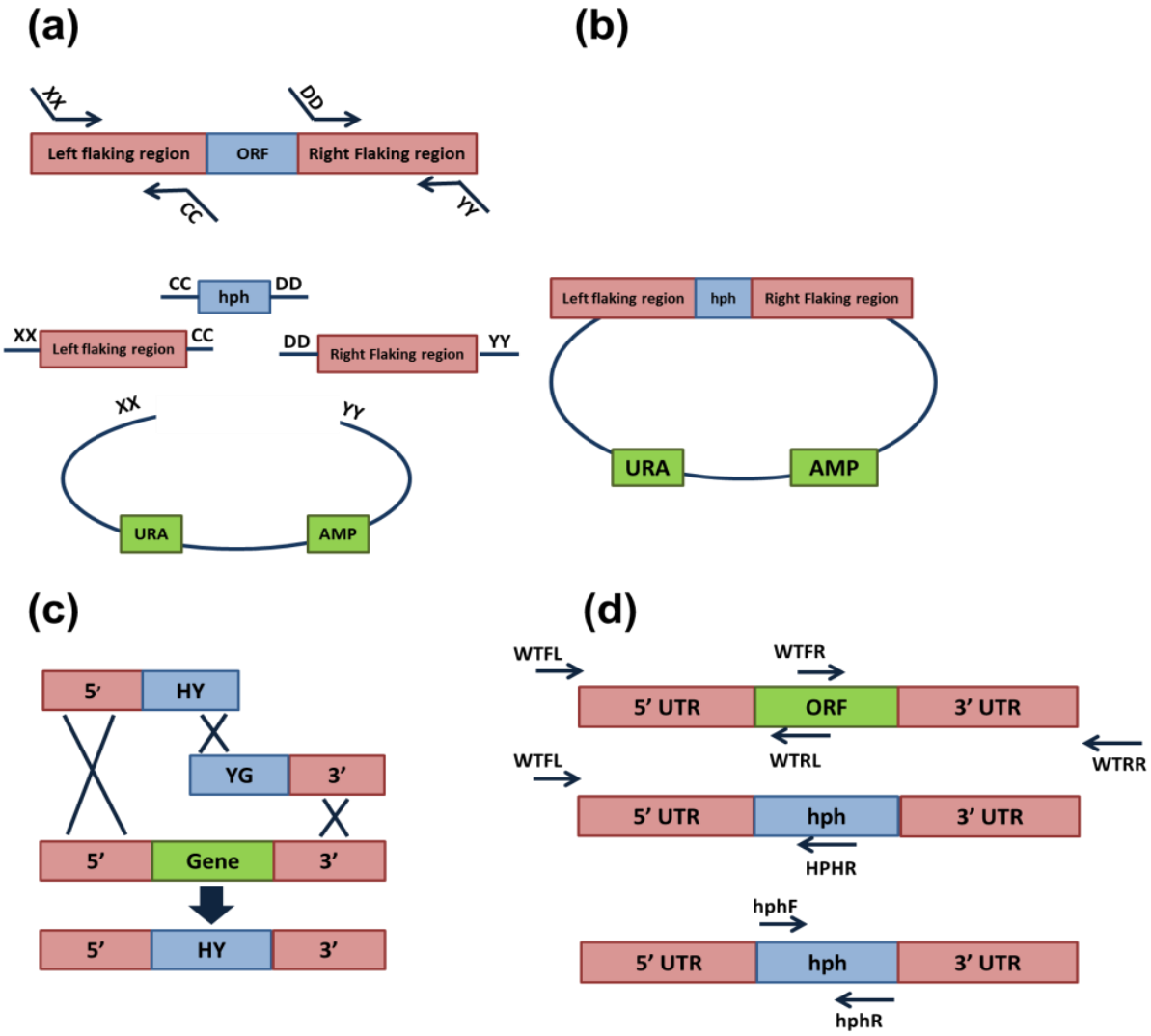


Figure 2.1 Knockout strategy for *Rhynchosporium commune* genes using yeast homologous recombination system. (a) (b) Creation of deletion cassettes by fusing the PCR products corresponding to the flanking regions for each of the genes in evaluation and (hph) selection marker assembled using yeast homologous recombination (c) Amplification of the deletion cassette using split marker strategy. Two PCR products corresponding to each flanking region fused to half of the hygromycin gene were used to transform *R. commune* conidia by electroporation. Homologous recombination is expected to occur between each flanking region and marker gene (d) Screening of *R. commune* transformants for gene disruption was carried out by genotyping PCR (gtPCR) after DNA extraction. Gene-specific primers were designed to confirm the presence of the (hph) gene in the correct place in the *R. commune* genome and the absence of the wild-type gene.

2.11.2 Electroporation of *R. commune* germinated conidia

R. commune conidia were harvested as described above. Conidia suspension was incubated in the dark on a shaker for ~24 h at 18°C to induce conidia germination. Germinated conidia were harvested by centrifugation at 1000 g for 10 min. Germinated conidia pellets were washed 2 times with 20 ml of 1 M sorbitol and centrifuged again using the same spinning conditions. 200 µl of germinated conidia were put into a pre-chilled electroporation cuvette, and DNA (2 µg of each split fragment in 20 µl) was added. Contents of the cuvette were mixed and incubated on ice for 20 min. Pulser (Bio-Rad) was set at 1.25 kV. Button was pressed once to pulse cells with time constant around 4.5 - 5.3 ms. After electroporation, 1 ml of ice-cold PDB/1 M sorbitol was added to the cuvette, solution was mixed and cells were transferred into a 25 ml of ice-cold PDB/1 M sorbitol, then incubated on a shaker overnight at 18°C. Transformed conidia were harvested by centrifugation at 1000 g for 10 min, and concentrated conidia suspension was plated onto selection agar medium containing hygromycine (Melford Laboratories Ltd) and ampicilline (Sigma), and incubated for two weeks. After 15 days, *R. commune* colonies were growing on CZV8CM agar media(Newton, 1989) containing ampicilline (50 µg/ml) and hygromycine (100 µg/ml). Simultaneously an aliquot of electroporated *R. commune* conidia was grown on control plates (without antibiotics) to test for fungal cell viability after electroporation. Once single colonies started growing on selective medium they were sub-cultured onto new selective plates.

2.11.3 Fungal DNA extraction

A small section of each of the *R. commune* colonies grown on selective medium was used for DNA extraction. The colony was suspended in 300 µl of SDS extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 200 mg of small glass beads and then disrupted in a tissue lyser (bead beater) (Qiagen) for 1 min at 30 s/cycle. Samples were briefly spun (16,000 x g for 15 s) and 150 µl of 3M sodium acetate, pH 5.5, was added and the samples incubated at -20°C for 10 min. Centrifugation was carried out at 16,000 g for 5

min, then supernatant was collected. 2.5 x sample volume of ethanol was added to the sample and subsequently spun at 16000 g for 30 min to pellet DNA. Supernatant was removed and pellet was washed with 150 µl of 70% ethanol. After centrifugation at 16000 x g for 2 min, supernatant was removed and the pellet was air dried. Pellet was re-suspended in 10 µl of elution buffer (EB).

2.11.4 Genotyping PCR strategy (gtPCR)

To test for positive transformants, the DNA samples were screened using four pairs of primers. First set of primers, wild type forward and wild type reverse primers for left flanking region (WTFL, WTRL) and second pair of primers, wild type forward and wild type reverse primers for right flanking region (WTFR, WTRR) were designed to amplify the flaking regions of the wild type gene (Fig. 2.1). The third pair of primers corresponding to wild type forward primer for left flanking region and hygromycin reverse primer (WTFL, HPHR) will give an amplicon if the wild-type gene has been replaced by the selection marker hygromycine (hph) (Fig. 2.1). To make sure that the lack of bands was not due to the absence of gDNA in the PCR tube, a set of actin primers (loading control) was included. In addition some amplifications were carried out using a fourth set of primers, hph forward and hph reverse (hphF, hphR) to amplify full length hygromycine gene (Figure 2.1).

Chapter 3. A new proteinaceous PAMP identified in Ascomycete fungi induces cell death in Solanaceae

3.1 Introduction

Recognition of conserved microbial elicitors, also known as PAMPS or MAMPs (Boller, 1995), initiates PTI in plants (Boller & Felix, 2009; Dodds & Rathjen, 2010). PAMPs are secreted by microorganisms or released from their cells by hydrolytic enzymes during interaction with the plant. They are evolutionarily conserved across classes of microbes and are important to the microbial lifestyle. Although a number have been identified the full repertoire of microbial PAMPs remains unknown.

PAMPs are recognized by plant cell surface localized immune receptors known as PRRs. Some PAMPs elicit defence responses in a wide range of plant species, while response to others is restricted to certain plant species, probably reflecting the presence of a particular PRR.

R. commune is a hemibiotroph with an extended asymptomatic phase (Avrova & Knogge, 2012). Following conidia germination and cuticle penetration *R. commune* hyphae spread between the epidermal cells (Jones & Ayres, 1974; Lehnackers & Knogge, 1990; Thirugnanasambandam *et al.*, 2011). Like several other important fungal pathogens of cereals, including *M. oryzae, Z. tritici and Parastagonospora nodorum, R. commune* belongs to the Ascomycota. This phylum also contains major pathogens of dicots, such as *B. cinerea* and *S. sclerotiorum,* as well as the model fungus *N. crassa.*

Sequencing of RNA from epidermal strips of barley leaves at 3 dpi with *R. commune* (Penselin *et al.*, 2016) followed by selection of sequences with BLAST matches to protein-coding genes from other fungi led to identification of an abundant transcript with the top match (*E*=1e-35) to the hypothetical protein MBM_09206 from *Marssonina brunnea* f. sp. 'multigermtubi' MB_m1, a fungal pathogen of poplar. The complete DNA sequence was

obtained by BLASTN search using this transcript sequence against the whole genome sequence of *R. commune* strain 13-13 (Penselin *et al.*, 2016).

QRT-PCR was used to obtain the expression profile of this *R. commune* gene in *in vitro*, pre-infection structures of conidia and germinated conidia, and during infection of barley leaves. Transcript abundance increased early during barley infection with *R. commune,* peaking at 3 dpi, during barley leaf cuticle penetration and the onset of apoplast colonisation, to over 40 times its level in conidia (Figure 3.1a, Figure 3.2). At this stage the transcript was 12-fold more abundant than that of fungal actin.

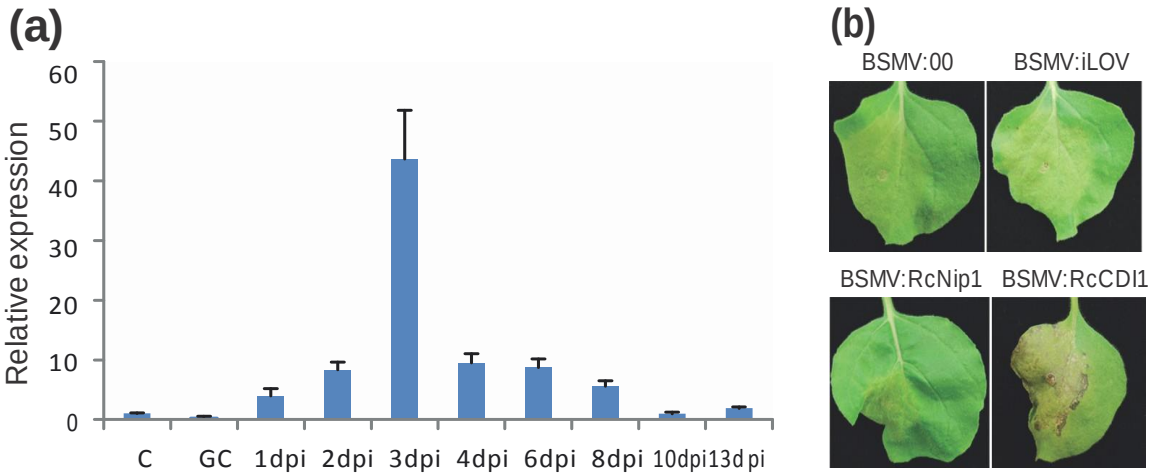


Figure 3.1 The *R. commune* gene *RcCDI1* upregulated during penetration of barley leaves, encodes a small secreted protein that induces cell death in nonhost plant *N. benthamiana*. (a) RcCDI1 transcript abundance in *R. commune* germinated conidia (GC) and at 1, 2, 3, 4, 6, 8, 10 and 13 days post-inoculation (dpi) of susceptible barley cv Optic with *R. commune* relative to its level in conidia (C), which was assigned the value 1.0. Error bars represent 95% confidence intervals calculated using three technical replicates for each sample within the RT-PCR assay. Assays repeated on three independent occasions, using leaf material from three independent infection time courses for RNA isolation and subsequent cDNA synthesis generated similar expression profiles. (b) Representative *N. benthamiana* leaves 6 days post agroinfiltration (dpi) with BSMV:00 (empty vector) and BSMV expressing iLOV, RcNip1and RcCDI1. This work was carried out by Dr. Anna Avrova and her group at The James Huton Institute and Dr. Kostya Kanyuka and his group at Rothamsted Research, prior to the beginning of my PhD. The Figure was generated using images kindly made availabe to me by Drs Avrova and Kanyuka.

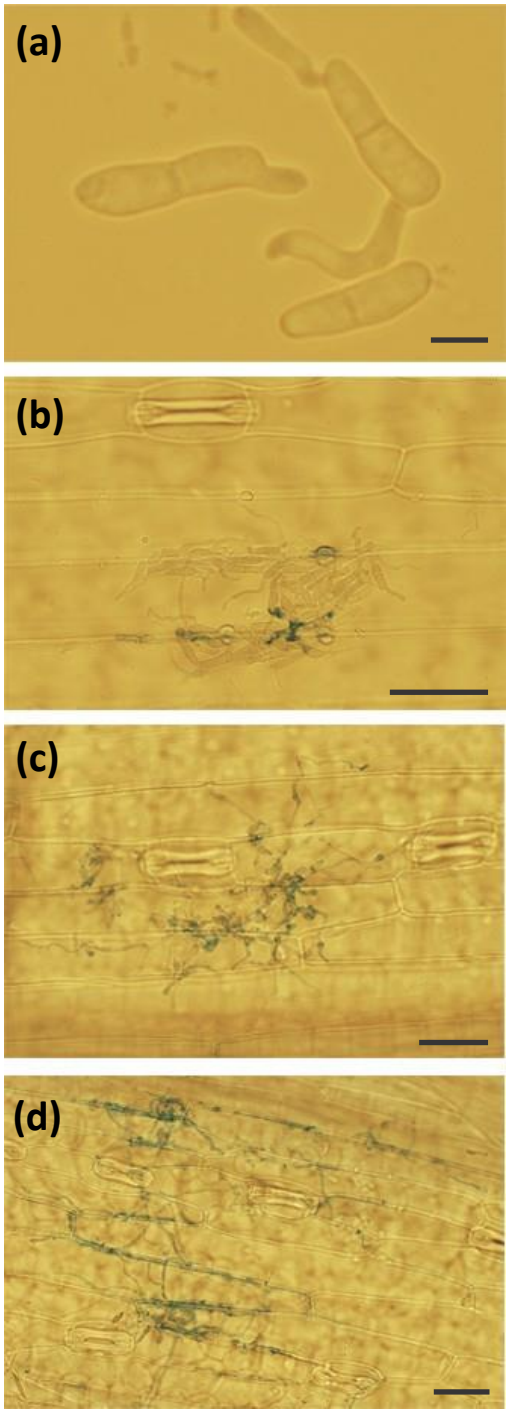


Figure 3.2 Trypan blue staining and microscopic assessment of *R. commune* infection stages on highly susceptible barley cv Optic. *R. commune* conidia were allowed to germinate in water for 24 h (a). At three days post inoculation (dpi) with a conidia suspension, germinating conidia (b) were visible on the leaf surface. At six dpi, infection hyphae (c) were observed spreading out from the infection point. At eight dpi, *R. commune* hyphae were observed aligning alongside the epidermal cell walls (d). Scale bars represent 10 µm for (a) and 40 µm for (b-d). The Figure was generated using images kindly made available to me by Dr Avrova.

Due to its expression profile, this was one of the first *R. commune* candidate effector proteins selected for functional characterisation using virus-mediated overexpression (VOX) in barley

leaves. The BSMV binary VOX vector system (Lee *et al.*, 2012) involves initial reconstitution and propagation of a recombinant BSMV in *N. benthamiana* following vector delivery via Agrobacterium-mediated leaf infiltration. Surprisingly, infiltration of *N. benthamiana* leaves with *A. tumefaciens* carrying a BSMV VOX vector expressing this candidate effector with endogenous signal peptide for secretion into the plant apoplast, induced strong cell death in inoculated leaves, and this prevented systemic virus spread (Figure 3.1b). At the same time, there was no cell death response to wild type (wt) BSMV or to BSMV expressing fluorescent protein iLOV (Chapman *et al.*, 2008), 2008) or *R. commune* avirulence protein RcNIP1 (Rohe *et al.*, 1995).

This led to a hypothesis that this protein, named RcCDI1 (Cell Death Inducing), is a PAMP recognised in the leaf apoplast of *N. benthamiana* by an unknown plant cell surface receptor. This protein was shown to be conserved across different Ascomycetes, with RcCDI1 homologues from *N. crassa, Z. tritici, M. oryzae, B. cinerea* and *S. sclerotiorum* also capable of inducing cell death in Solanaceae but not in other dicots or monocots.

3.2 Results

3.2.1 *R. commune* RcCDI1 encodes a small secreted protein inducing cell death in a nonhost plant *N. benthamiana*

Sequence analysis of the *R. commune* candidate effector gene revealed that it does not have any introns and codes for a 200 amino acid secreted protein, containing 4 cysteine residues. The function of RcCDI1 remains undescribed and there are no annotated domains assigned for this protein (Figure 3.3).

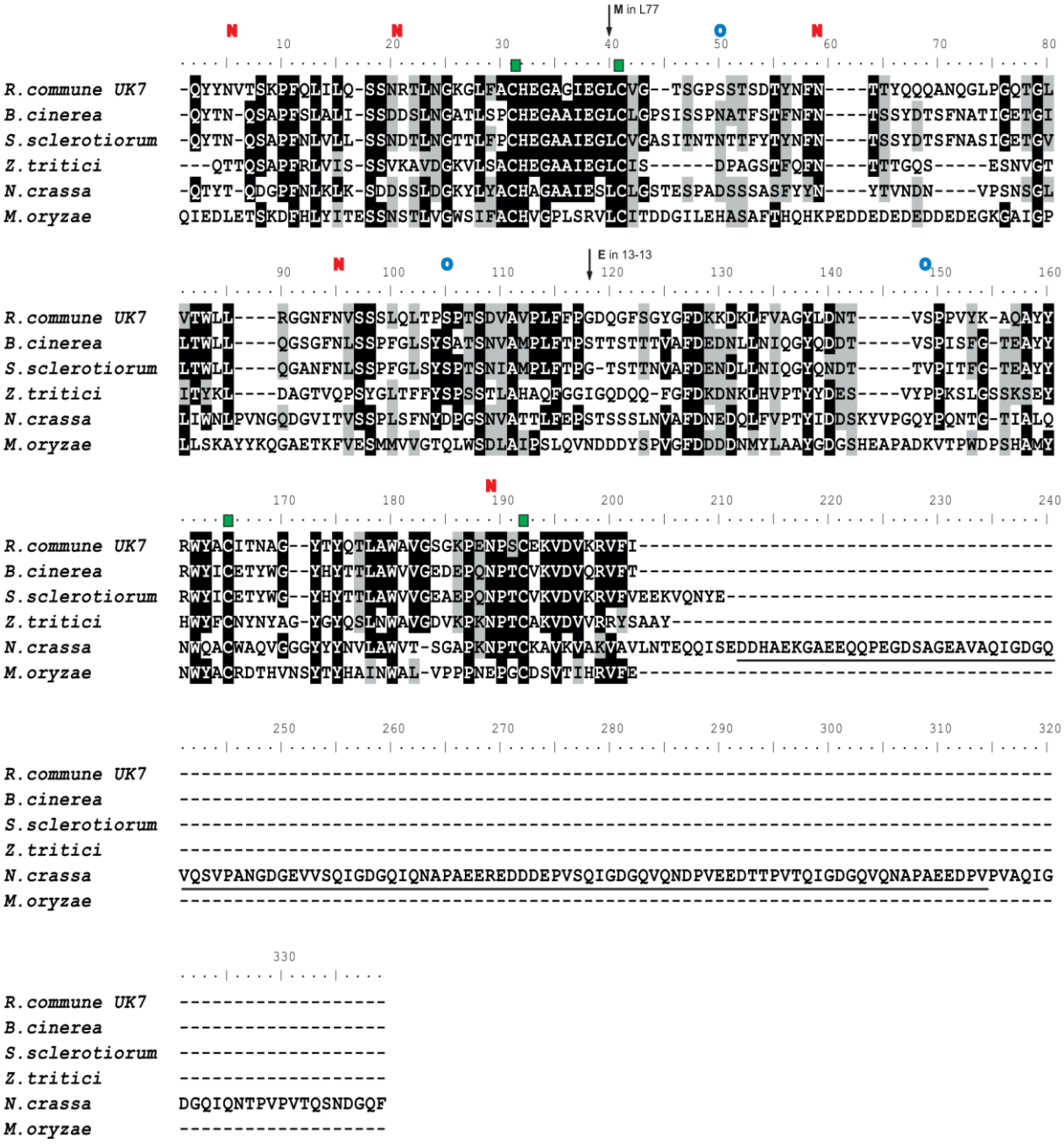


Figure 3.3 ClustalW alignment of mature protein sequences of *R. commune* CDI1 with its homologues from other Ascomycete fungi. BC1G_01016, *Botrytis cinerea* B05.10, (XP_001560184); SS1G_09232, *Sclerotinia sclerotiorum* 1980 UF-70 (XP_001589511); MYCGRDRAFT_111505, *Zymoseptoria tritici* IPO323, (XP_003847964); *Neurospora crassa* OR74A (EAA29378) and MGG_15553, *Magnaporthe oryzae* 70-15 (XP_003713665.1). Red N and blue O mark the predicted N-glycosylation and O-glycosylation sites, respectively, in RcCDI1. Green squares indicate the conserved cysteine residues. The K+-dependent Na+/Ca+ exchanger domain in the *N. crassa* protein is underlined.

To further confirm that the observed cell death was caused by RcCDI1, the full length and truncated (lacking a signal peptide) versions of *RcCDI1* were also transiently expressed in *N. benthamiana* leaves as a C-terminal mRFP fusion protein from a non-viral, conventional

binary vector pK7RWG2, producing fusion proteins of expected size (Figure 3.4 a,b,c). Once again, full-length RcCDI1 induced strong cell death, while RcCDI1 lacking a signal peptide did not. Moreover, as a control the *P. infestans* apoplastic protease inhibitor EpiC1 was expressed with a signal peptide and C-terminal mRFP fusion (SP-PiEpiC1) and also failed to induce cell death (Figure 3.4 d,e). These data indicated that cell death induction by RcCDI1 in *N. benthamiana* leaves requires protein targeting to the apoplast.

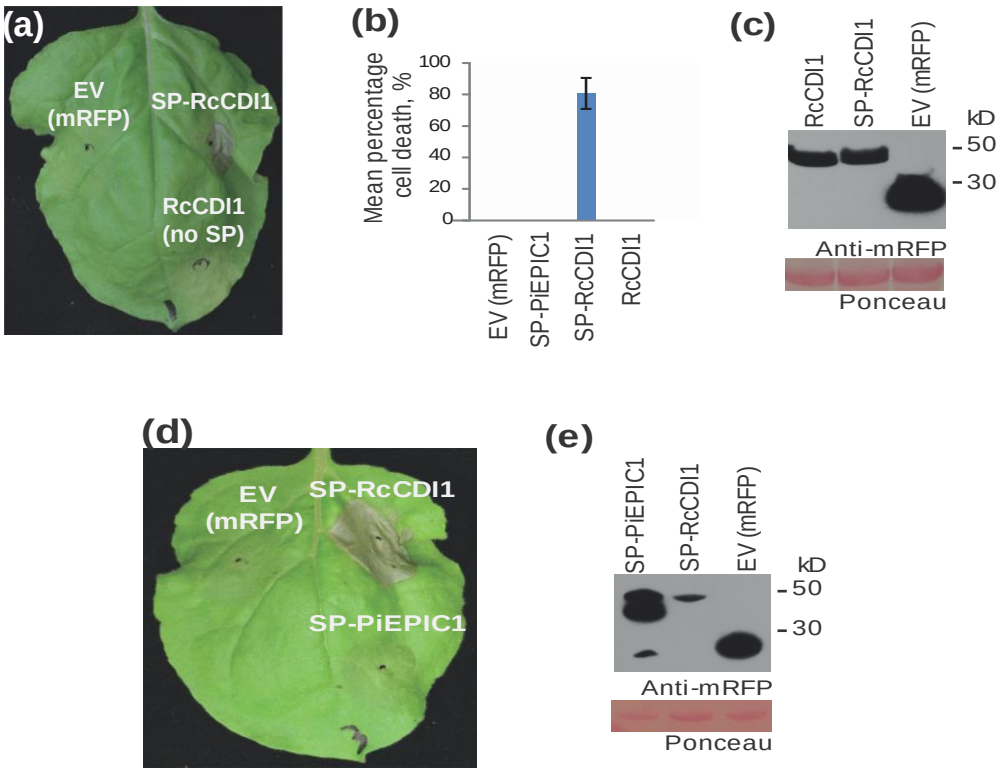
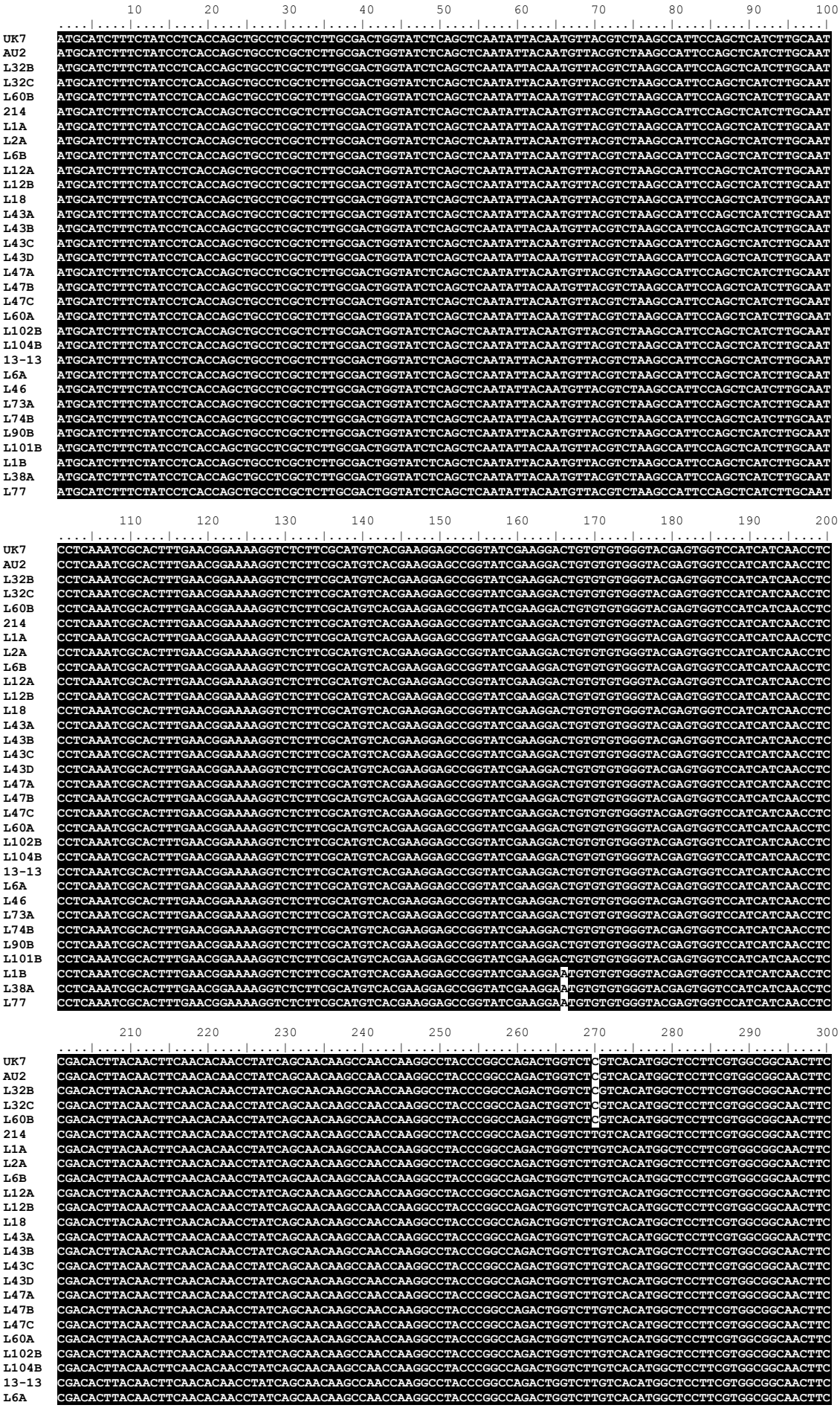


Figure 3.4 The *R. commune* gene RcCDI1 expressed with and without signal peptide (SP) induces cell death in *N. benthamiana* (b) The percentage of infiltration sites developing a clear cell death in *N. benthamiana* leaves at 6 dpi mediated by a pK7RWG2 vector control (EV) expressing mRFP or RcCDI1 with C-terminal mRFP fusion with or without signal peptide. Experiments were repeated at least three times, each with no less than eight plants, and error bars indicate ± SD. (a) Representative *N. benthamiana* leaf 6 dpi using pK7RWG2 constructs expressing the RcCDI1 with C-terminal mRFP fusion with (SP-RcCDI1) and without (RcCDI1) signal peptide. (c), (e) Immunoblot of proteins from *N. benthamiana* leaves transiently expressing the indicated proteins with C-terminal mRFP fusion from a pK7RWG2 vector. (d) Representative *N. benthamiana* leaf 6 dpi using pK7RWG2 constructs expressing the RcCDI1 with C-terminal mRFP fusion with signal peptide (SP-RcCDI1) and the *P. infestans* apoplastic protease inhibitor EpiC1 with C-terminal mRFP fusion with signal peptide (SP-PiEpiC1). SP-PiEpiC1 construct was provided by Shumei Wang from The James Hutton Institute.

3.2.2 All three alleles of *RcCDI1* induce cell death in *N. benthamiana*

Amplification and sequencing of *RcCDI1* from 32 isolates of *R. commune* confirmed all tested isolates to contain *RcCDI1* and revealed 6 different single nucleotide polymorphisms (SNPs) in this gene sequence (Figure 3.5). Only 2 out of 6 SNPs led to nonsynonymous substitutions (Figure 3.3, 3.5, Table 3.1). The RcCDI1-LG allele is the most common present in 22 out of 32 tested isolates including UK strain UK7 and an Australian isolate AU2 (Table 3.1). Seven isolates including UK strain 13-13 had a single nucleotide polymorphism (SNP) at position 371, leading to change in amino acid from glycine to glutamic acid (Figure 3.5, Table 3.1). The remaining 3 isolates, including strain L77 had a SNP at position 166, leading to change from leucine to methionine (Figure 3.5, Table 3.1). All three alleles of RcCDI1 containing signal peptide were transiently expressed in *N. benthamiana* leaves as C-terminal mRFP fusion proteins. All produced proteins of expected size (Figure 3.6c) and induced strong cell death (Figure 3.6a,b).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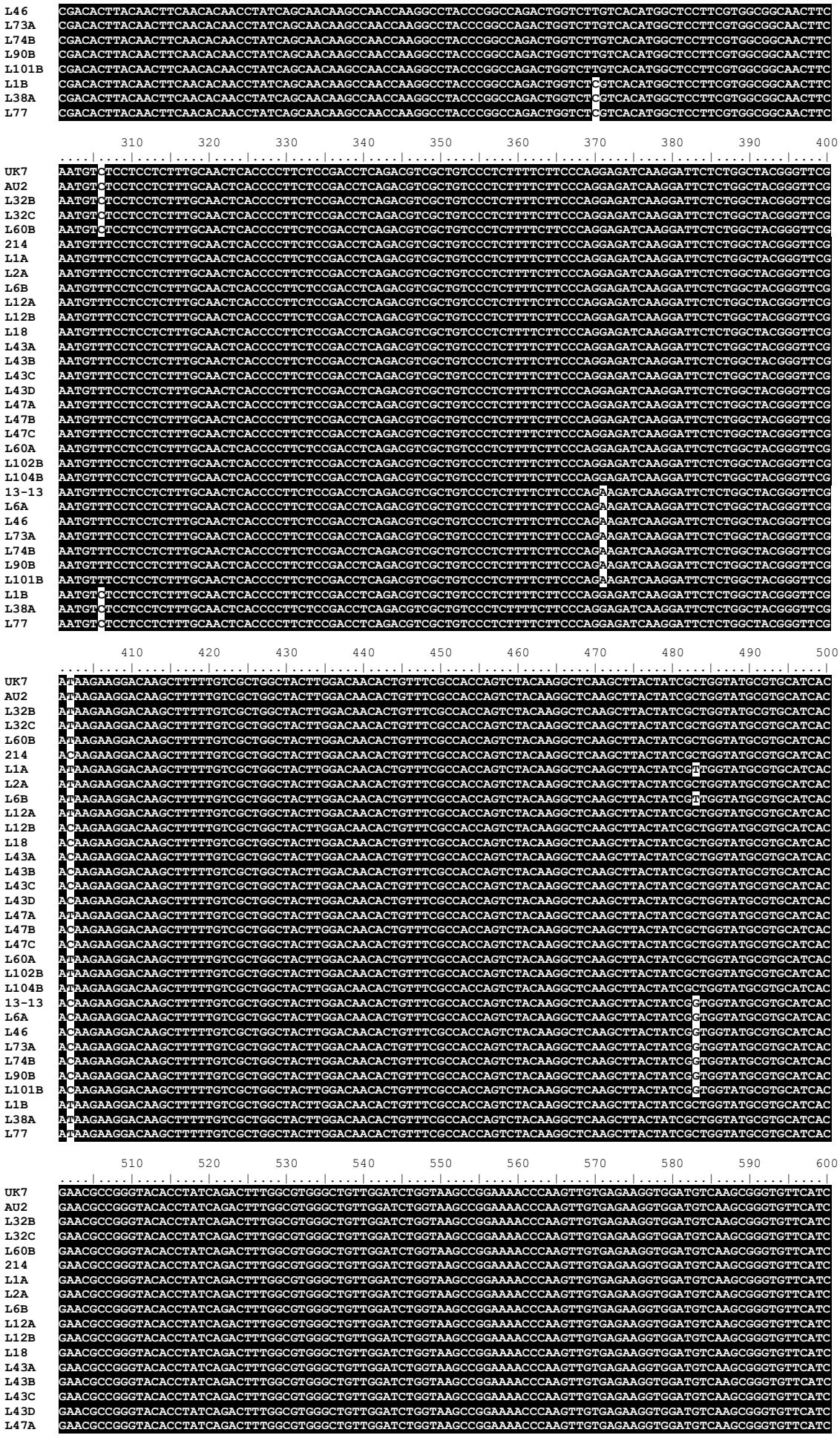
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Figure 3.5 ClustalW alignment of DNA sequences of *R. commune CDI1*. 32 strains showing 6 single nucleotide polymorphisms (SNPs) at positions 166, 270, 306, 371, 402 and 483.

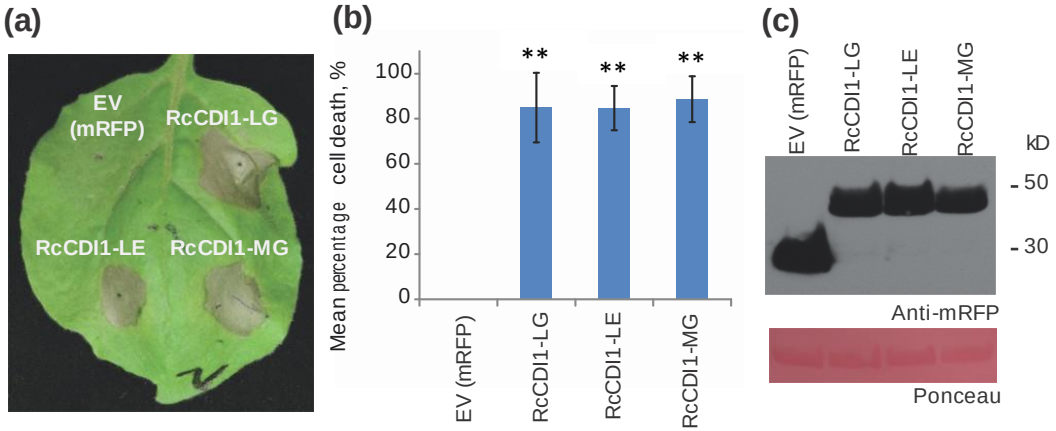

Figure 3.6 All three alleles of RcCDI1 induce cell death in *N. benthamiana*. (a) Representative *N. benthamiana* leaf 6 days post agroinfiltration (dpi) using pK7RWG2 constructs expressing 3 different alleles of *RcCDI1* (*RcCDI1*-LG, *RcCDI1*- LE and *RcCDI1*-MG) with C-terminal mRFP fusion and signal peptide. (b) The percentage of infiltration sites developing a clear cell death in *N. benthamiana* leaves at 6 dpi mediated by a pK7RWG2 vector control (EV) expressing mRFP or 3 different alleles of RcCDI1 with C-terminal mRFP fusion with signal peptide. Experiments were repeated at least three times, each with no less than eight plants, and error bars indicate ± SD. (c) Immunoblot of proteins from *N. benthamiana* leaves transiently expressing the indicated proteins with C-terminal mRFP fusion from a pK7RWG2 vector. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; $^{**}P \leq 0.01$.

Table 3.1 Distribution of *RcCDI1* alleles in different *R. commune* isolates

Position	RcCDI1allele	*R. commune* isolates
	L-G	UK7, AU2, 214, L1A, L2A, L6B, L12A, L12B, L18, L32B, L32C, L43A, L43B, L43C, L43D, L47A, L47B, L47C, L60A, L60B, L102B, L104B
371	L-E	13-13, L6A, L46, L73A, L74B, L90B, L101B
166	M-G	L1B, L38A, L77

3.2.3 *RcCDI1* gene knockout

Deletion cassette containing the left and right flanking regions fused to the selection marker hygromycine (hph) was produced for the *R. commune* candidate gene *RcCDI1*. Each of the flaking regions, which are around 1 Kb in length, and the hygromycine gene (1.2 Kb) were amplified using primers with extensions with homology to the linearised vector PRS426 (Figure 3.7a). The two flaking regions, hygromycin gene and linearised vector were combined in *S. cerevisae* by homologous recombination (Figure 2.1a,b). The deletion cassettes were used as a template for the PCR amplifications of two overlapping products as part of the split marker strategy (Figure 3.7b). The two resulting products were used for *R. commune* transformation during which three recombination events are expected to occur for the integration of the deletion cassette and replacement of the gene of interest with the selection marker. Two of these recombination events occur between the flaking regions in the PCR products and the genomic DNA and one for the hygromycin gene (Figure 2.1c).

R. commune transformed colonies were grown on medium with hygromycin selection (Figure 3.7d). DNA extraction was carried out for the putative transformants to check for the right insertion of the deletion cassette in the genome and the achievement of a knockout. The majority of the colonies showed the presence of the wild type gene using the first (WTFL, WTRL) or the second (WTFR, WTRR) primer set and also the presence of the hygromycin gene using the fourth primer set (hphF, hphR) (Figure 3.7c,d). After checking for the insertion of the hygromycin gene in the right genomic location, using the third primer set (WTFL,

HPHR), leading to the replacement of *RcCDI1* by the selection marker, no amplification was obtained for any of the transformants, which means that non-homologous recombination occurred leading to insertion of the deletion cassette in a random place in the *R. commune* genome. After checking 98 colonies no *RcCDI1* deletion transformants were obtained. Actin amplicon was amplified for all samples as a positive control (Figure 3.7c).

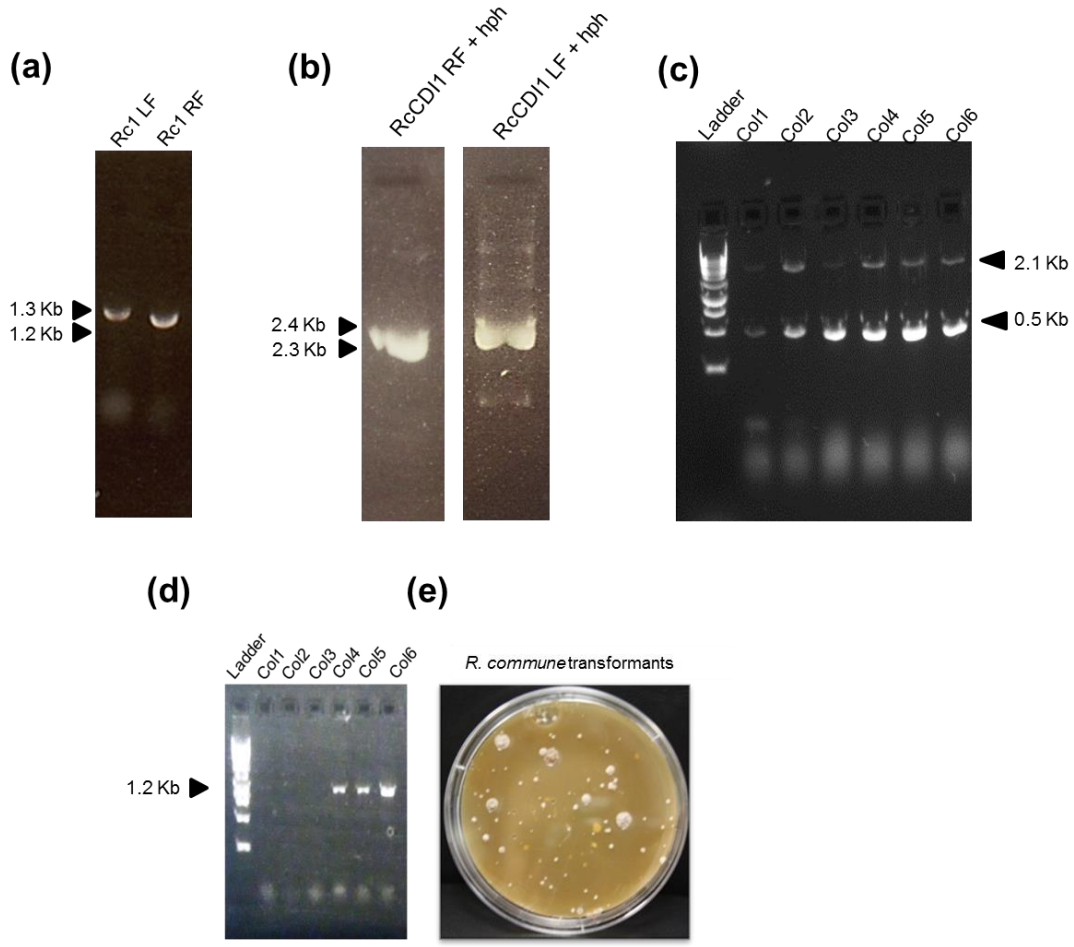

Figure 3.7 Generation of *R. commune RcCDI1* transformants using yeast homologous recombination system. (a) Agarose gel showing the amplification products for left (LF) and right (RF) flanking regions of *RcCDI1* gene with extensions specific to the yeast linearized vector PRS426 and hygromycine selection marker. (b) Amplification of the deletion cassette as two overlapping fragments containing left or right flaking region of the gene fused to one part of the hygromycine gene (c) Agarose gel showing the amplification of the 2.1 kb wild type LF region of *RcCDI1* for the six tested *R. commune* transformants. Amplification of 0.5 kb actin gene fragment was used as a positive control. (d) Agarose gel showing the amplification of full length hygromycine gene for three out of six colonies tested. (e) *R. commune* transformants growing on selection media containing hygromycine after transformation.

3.2.4 Ascomycete fungi contain homologues of RcCDI1 which also induce cell death in *N. benthamiana*

Querying RcCDI1 protein sequence against the NCBI protein database using BLASTP resulted in identification of homologous proteins in other Ascomycetes, including pathogens of monocots such as *Z. tritici*, *B. graminis*, *M. oryzae* and of dicots such as *B. cinerea*, *S. sclerotiorum;* as well as pathogens of nematodes and arthropods, birds and mammals, and saprophytes including *N. crassa* (Figure 3.3, Figure 3.8, Table 3.2). No homologues of RcCDI1 were identified in fungal phyla other than Ascomycetes, or in oomycetes or bacteria.

All RcCDI1 homologues found in different Ascomycete fungi were of comparable size, except for the *N. crassa* homologue that was noticeably longer due to the presence of an additional putative K+-dependent Na+/Ca+ exchanger domain (*E* = 6.74e-03) identified at the C-terminus (Figure 3.3). All RcCDI1 homologues contained the four conserved cysteine residues (Figure 3.3).

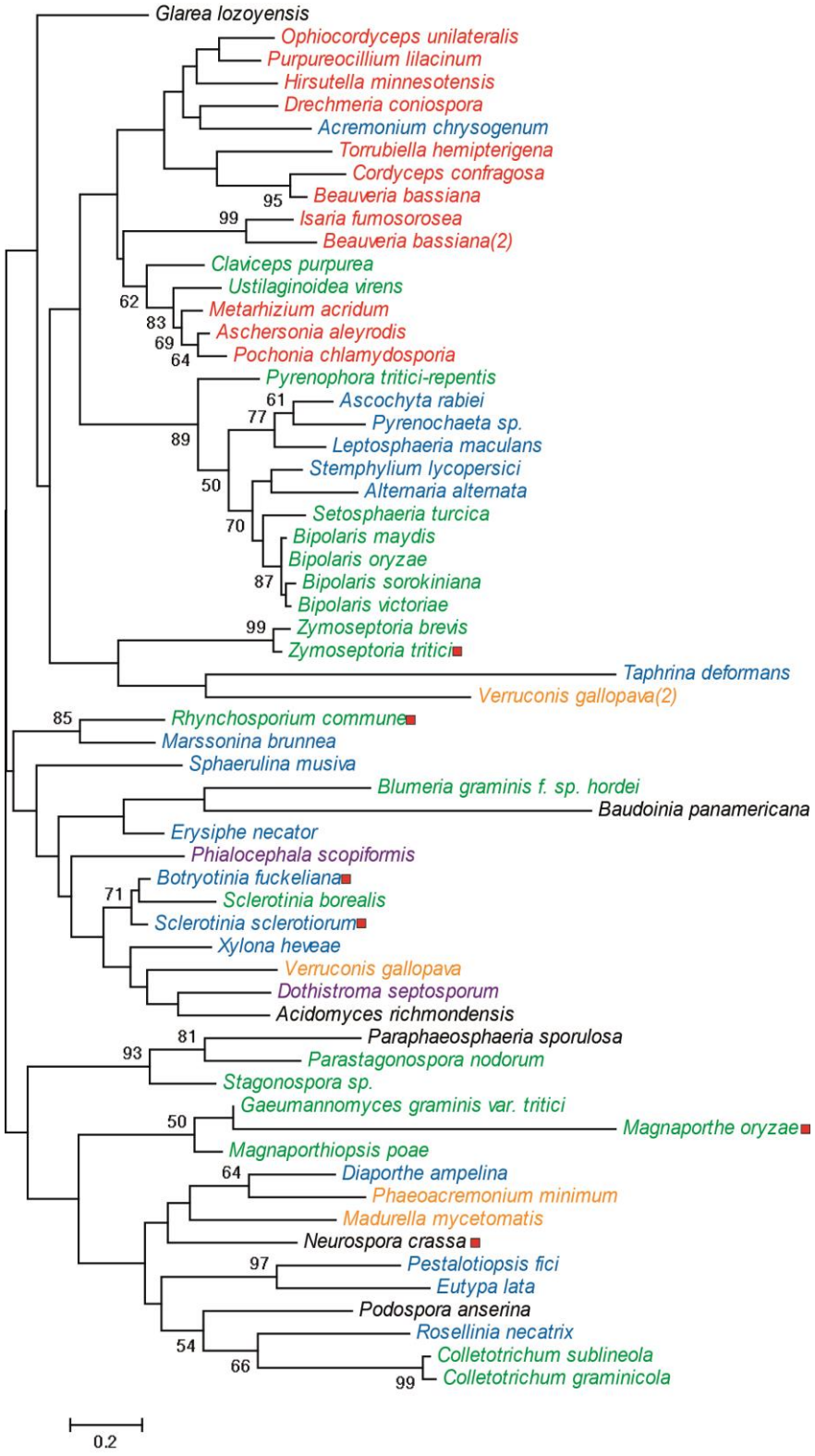

Figure 3.8 The CDI1 family is distributed widely across the Ascomycetes. Phylogeny of RcCDI1 and 60 related sequences from selected species. Bootstrap support values (> 50 %) from 1000 replicates are shown at the nodes. The names of fungal pathogens or endophytes of cereals and grasses, dicots, conifers, nematodes and arthropods, and birds and mammals are in green, blue, purple, red and orange, respectively. The names of saprophytes are in black. The proteins shown to induce cell death in *N. benthamiana* are indicated by a red square. Accessions of protein sequences are shown in Table 3.2.

Table 3.2 RcCDI1homologues from different fungi

Fungal species	Accession number	E value	% similar amino acids	% identical amino acids	Host species
Marssonina brunnea f. sp. 'multigermtubi' MB_m1	XP_007297095.1	2e-72	69	57	Poplar
Phialocephala scopiformis	XP_018078062.1	8e-64	70	54	conifer needle endophyte
Botrytis cinerea B05.10	XP_001560184.1	7e-57	65	51	multiple dicot species
Sclerotinia borealis F-4157	ESZ90221.1	3e-54	61	49	barley, rye, wheat
Sclerotinia sclerotiorum 1980	XP_001589511.1	9e-54	62	49	multiple dicot species
Xylona heveae TC161	XP_018192293.1	2e-52	59	43	endophyte of rubber trees
Ascochyta rabiei	KZM24312.1	3e-49	59	45	chickpea, common bean
Verruconis gallopava	XP_016210220.1	3e-48	61	46	human, saprophytic infection in immunosup pressed host
Erysiphe necator	KHJ32579.1	7e-45	62	43	Grapevine
Aschersonia aleyrodis RCEF 2490	KZZ93700.1	2e-43	58	42	Insects
Metarhizium acridum CQMa 102	XP_007815623.1	1e-42	57	43	Insects
Sphaerulina musiva SO2202	XP_016759421.1	1e-41	60	43	Poplar
Glarea lozoyensis ATCC 20868	XP_008082695.1	9e-41	59	42	Saprophyte
Claviceps purpurea 20.1	CCE32946.1	1e-39	60	43	cereals and grasses
Leptosphaeria maculans JN3	XP_003845256.1	5e-39	60	42	Brassicas and crucifers
Pyrenochaeta sp. DS3sAY3a	OAL52266.1	2e-38	57	40	Dicots
Torrubiella hemipterigena	CEJ93784.1	4e-38	57	43	Leafhopper
Pochonia	XP_018136634.1	9e-38	58	43	Nematodes

chlamydosporia 170					
Ustilaginoidea virens	KDB13604.1	9e-38	56	40	Rice
Cordyceps confragosa RCEF 1005	OAA78637.1	1e-37	59	42	insects and other arthropods
Beauveria bassiana D1-5	KGQ10086.1	1e-37	59	41	Arthropods
Stagonospora sp.	OAL01824.1	3e-37	57	39	Cereals
Drechmeria coniospora	KYK60555.1	8e-37	57	42	Nematodes
Diaporthe ampelina	KKY39290.1	2e-36	57	44	Grapevine
Bipolaris maydis ATCC 48331	XP_014081220.1	2e-36	54	42	Maize
Bipolaris sorokiniana ND90Pr	XP_007695397.1	6e-36	54	42	Cereals
Bipolaris victoriae	XP_014551974.1	7e-36	54	42	Oats
Bipolaris oryzae ATCC 44560	XP_007689641.1	1e-35	54	42	Rice
Hirsutella minnesotensis 3608	KJZ74748.1	5e-34	60	44	soybean cyst nematode
Dothistroma septosporum NZE10	EME45809.1	7e-34	55	44	Conifers
Acidomyces richmondensis	KXL41726.1	1e-33	57	42	Saprophyte
Ophiocordyceps unilateralis	KOM17321.1	4e-33	58	42	Insects
Zymoseptoria brevis	KJX92807.1	7e-33	57	44	Barley
Stemphylium lycopersici	KNG45866.1	8e-33	54	38	tomato, capsicum, carnation, papaya
Zymoseptoria tritici IPO323	XP_003847964.1	2e-32	57	44	Wheat
Pyrenophora tritici-repentis Pt-1C-BFP	XP_001932972.1	7e-32	55	37	small grain cereals, grasses
Madurella mycetomatis	KXX73716.1	8e-32	57	40	Humans
Isaria fumosorosea ARSEF 2679	OAA62297.1	2e-31	56	41	Arthropod
Purpureocillium lilacinum	XP_018174993.1	2e-29	58	41	insects, fungi, nematodes as well as a saprophyte
Setosphaeria turcica Et28A	XP_008020997.1	2e-29	52	41	maize and sorghum

Colletotrichum sublineola	KDN72286.1	9e-29	53	41	wild rice and sorghum.
Acremonium chrysogenum ATCC 11550	KFH42584.1	1e-28	52	37	cassava saprophyte
Beauveria bassiana ARSEF 2860	XP_008601187.1	3e-28	54	38	Insects
Magnaporthiopsis poae ATCC 64411	KLU87402.1	3e-27	51	39	Grasses
Paraphaeosphaeria sporulosa	XP_018031983.1	2e-26	51	36	Endophyte
Gaeumannomyces graminis var. tritici R3-111a-1	XP_009217977.1	4e-26	52	39	Wheat
Alternaria alternata	XP_018386080.1	8e-26	52	34	multiple dicot species
Phaeoacremonium minimum UCRPA7	XP_007910801.1	2e-25	52	40	woody plants and humans
Neurospora crassa OR74A	XP_958614.3	6e-25	52	38	Saprophyte
Taphrina deformans PYCC 5710	CCG80990.1	3e-24	50	40	Peach
Parastagonospora nodorum SN15	XP_001792149.1	9e-24	51	36	Wheat
Blumeria graminis f. sp. hordei DH14	CCU82201.1	1e-23	50	34	Barley
Colletotrichum graminicola	CAQ16238.1	5e-23	51	39	Cereals
Magnaporthe oryzae 70-15	XP_003713665.1	3e-20	44	32	Rice
Rosellinia necatrix	GAP89223.1	6e-19	50	37	apple, grapevine, tea
Podospora anserina S mat+	XP_001908074.1	8e-17	49	33	Saprophyte
Baudoinia panamericana UAMH 10762	XP_007674755.1	2e-15	46	34	Saprophyte
Verruconis gallopava	XP_016217265.1	5e-14	48	30	mammals and birds
Pestalotiopsis fici W106-1	XP_007830922.1	5e-14	47	31	endophyte of tea
Eutypa lata UCREL1	EMR71622.1	2e-12	47	33	grapevine and apricot

We assessed whether *RcCDI1* homologues from other Ascomycetes also induce cell death in *N. benthamiana*. For this, *RcCDI1* and its homologues from *N. crassa, Z. tritici* and *M. oryzae*, PiINF1 (a known elicitor of cell death from *P. infestans*) and another *R. commune* putative secreted protein Rc2, used as a negative control, were produced with a C-terminal V5 epitope tag (Southern *et al.*, 1991) in *P. pastoris* using the modified pPICZαA vector targeting expressed proteins for secretion into the culture medium (Figure 3.9a). PiINF1, RcCDI1, NcCDI1 were produced at high levels, and following electrophoretic analysis of the *P. pastoris* CS they were clearly visible as discrete protein bands of ~13, 42, 58 kDa, respectively, on SYPRO Ruby-stained polyacrylamide gels (Figure 3.9a). Rc2 was detected as a strong band of ~14 kDa on an immunoblot (Figure 3.8a). ZtCDI1 and MoCDI1 were produced at lower levels, and were detected as less strong ~30 and 38 kDa protein bands, respectively, on an immunoblot (Figure 3.9a). The *P. pastoris* CS containing ZtCDI1 and MoCDI1 were therefore concentrated ~20 fold before testing the effect of these proteins by infiltration into *N. benthamiana* leaves. While concentrating the *P. pastoris* CS, we replaced YPD (a nutrient rich medium for yeast growth) with HNT buffer to avoid increased concentration of *P. pastoris* secreted proteins, as well as salts and peptides present in YPD.

Protein sequences of Rc2, RcCDI1, NcCDI1, ZtCDI1 and MoCDI1 suggest that the molecular weights of the V5-tagged mature proteins should be 7.8, 23, 35.5, 20.8 and 24.1 kDa, respectively, whereas immunoblot (Figure 3.9a) revealed higher molecular weight for each of these fungal proteins. Heterologous expression of proteins in *P. pastoris* allows correct post-translational modifications, including glycosylation and correct folding of cysteine-rich proteins. Bioinformatic analysis identified a potential N- glycosylation site in Rc2 and several potential glycosylation sites in RcCDI1 and its homologues from other fungal species (Figure 3.3, Figure 3.9a, Table 3.3). Therefore, the slower migration of proteins observed in a gel than expected based on their predicted molecular weights, is likely attributed to glycosylation.

Infiltration of *P. pastoris* CS containing PiINF1 as well as those containing RcCDI1 homologues from *R. commune*, *N. crassa*, *Z. tritici* and *M. oryzae* into the apoplastic space of *N. benthamiana* leaves induced a strong cell death, while infiltration of *N. benthamiana* leaves with CS of *P. pastoris* expressing a V5 tag alone or Rc2-V5 did not induce cell death (Figure 3.9b). More closely related RcCDI1 homologues from *B. cinerea* and *S. sclerotiorum* expressed with a signal peptide in *N. benthamiana* using the binary vector pK7RWG2 also induced strong cell death (Figure 3.10).

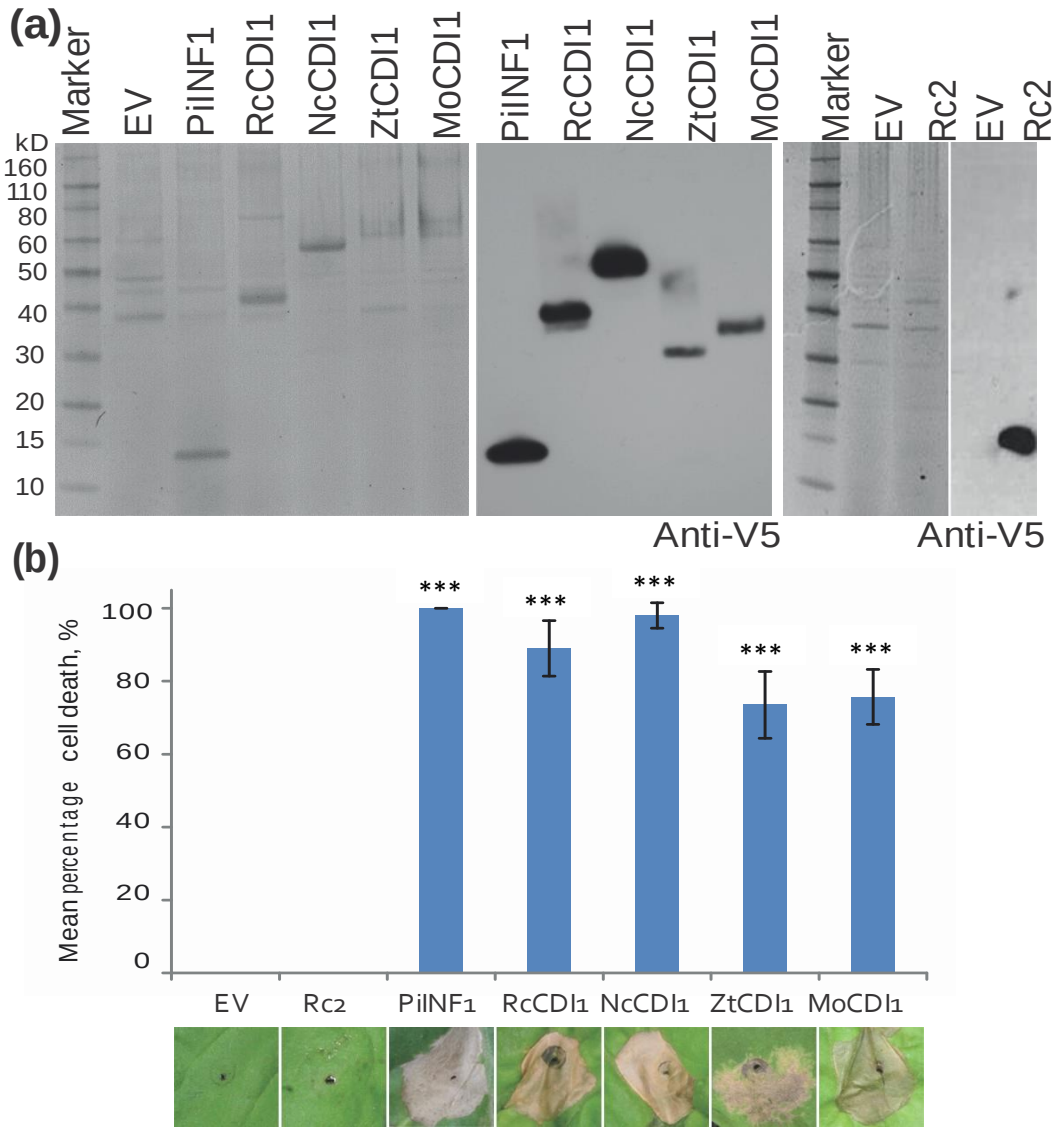

Figure 3.9 RcCDI1 homologues from *N. crassa*, *Z. tritici* and *M. oryzae* induce cell death in *N. benthamiana*. (a) SYPRO Ruby-stained SDS-PAGE and immunoblot using anti-V5 antibody of culture supernatant (CS) of *P. pastoris* strain GS115 expressing V5 (EV), *P. infestans* elicitin PiINF1-V5 (PiINF1), CDI1 from *R. commune* (RcCDI1), and its homologues from *N. crassa* (NcCDI1), *Z. tritici* (ZtCDI1) and *M.oryzae* (MoCDI1). SYPRO Ruby-stained SDS-PAGE and immunoblot of CS of *P. pastoris* strain GS115 expressing V5 (EV) and *R. commune* protein Rc2. (b) The percentage of

infiltration sites developing cell death in *N. benthamiana* leaves at two days post infiltration (dpi) with CS of *P. pastoris* expressing V5 (EV), Rc2, PiINF1-V5, RcCDI1-V5, NcCDI1-V5, concentrated ZtCDI1-V5 and MoCDI1-V5. Photographs of typical infiltration zones are presented in the panel beneath. Experiments were repeated at least two times, each with no less than five plants, and error bars indicate ± SD. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; ***$P \leq 0.001$.

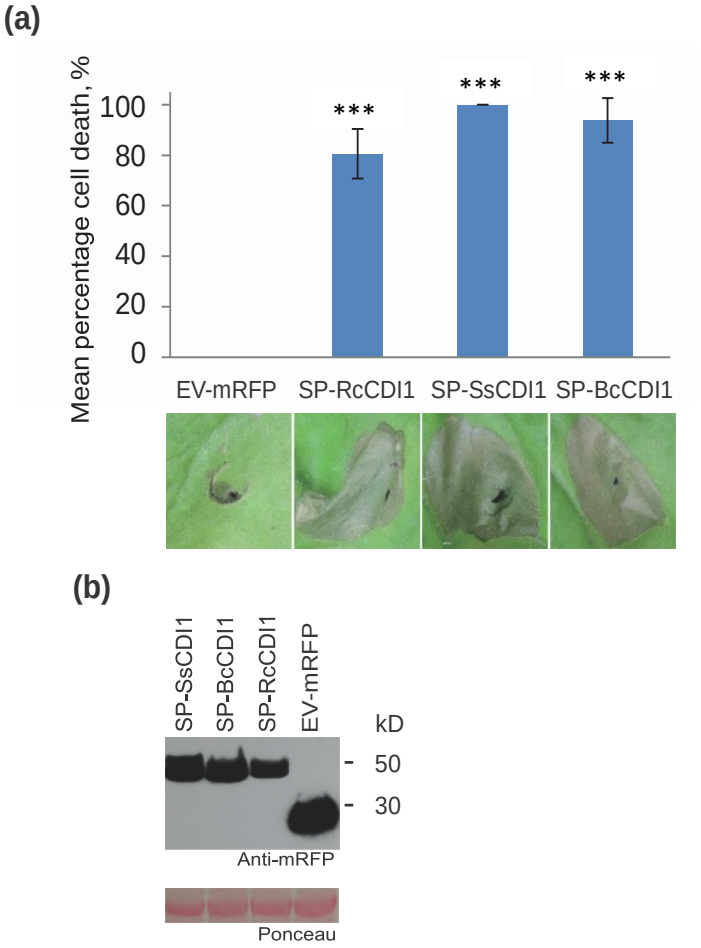

Figure 3.10 RcCDI1 homologues from *S. sclerotiorum* and *B. cinerea* induce cell death in *N. benthamiana*. (a) The percentage of infiltration sites developing cell death in *N. benthamiana* leaves at 7 days post infiltration (dpi) using pK7RWG2 constructs encoding mRFP (EV-mRFP) and the CDI1 homologues from *R. commune* (SP-RcCDI1), *S. sclerotiorum* (SP-SsCDI1) and *B. cinerea* (SP-BcCDI1) with native signal peptides. Photographs of typical infiltration zones are presented in the panel beneath. Experiments were repeated at least two times, each with no less than five plants, error bars indicate ± SD. (b) Immunoblot of proteins from *N. benthamiana* leaves transiently expressing the indicated proteins with C-terminal mRFP fusion from a pK7RWG2 vector. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; ***$P \leq 0.001$.

Table 3.3 Number of glycosylation sites in RcCDI1 and its homologues.

Protein name	Number of N-glycosylation sites	Number of O-glycosylation sites
RcCDI1	5	3
NcCDI1	1	1
ZtCDI1	1	0
MoCDI1	1	0
SsCDI1	7	0
BcCDI1	4	2

3.2.5 The RcCDI1 protein induces cell death in Solanaceae but not in other dicots or monocots

To allow quantification of the RcCDI1 or pINF1 protein produced by *P. pastoris*, they were purified from the *P. pastoris* CS using an Anti-V5 Affinity Gel (Figure 3.11). Purified RcCDI1-V5 was tested for cell death activity by infiltrating 100 pM to 1 µM protein solution into the apoplastic space of *N. benthamiana* leaves. RcCDI1-V5 induced cell death in *N. benthamiana* two days after protein infiltration, with 1 nM being the lowest concentration of RcCDI1-V5 triggering cell death (Figure 3.12). While 1 µM solution of RcCDI1consistently induced cell death each time it was infiltrated into *N. benthamiana* leaves, 1 nM solution of RcCDI1caused cell death in only one out of six infiltrated *N. benthamiana* leaves, suggesting that at this concentration there is barely sufficient protein reaching the putative cell surface receptor in the apoplast to trigger the visible, confluent cell death response.

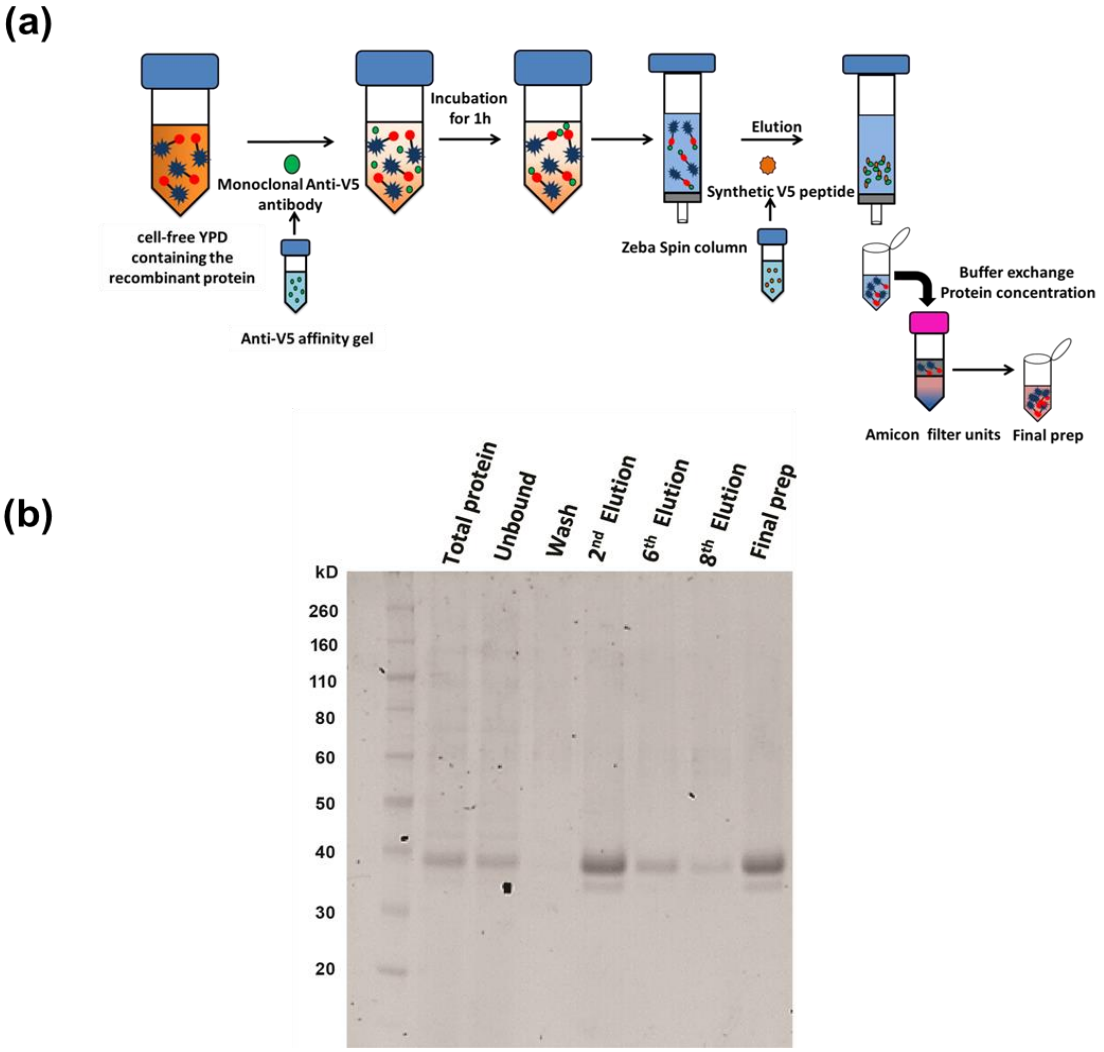

Figure 3.11 RcCDI1-V5 protein purification. (a) cell-free YPD containing the recombinant protein is incubated for 1 h with the Anti-V5 affinity gel. At this stage the monoclonal Anti-V5 antibody binds the V5-tagged recombinant protein. Sample is poured onto Zeba Spin column and is washed 4 times with TBS. The bound recombinant protein can then be eluted with synthetic V5 peptide in TBS competing for binding to the antibody. The unbound recombinant protein is washed from the column and collected. HNT buffer was preferable over TBS buffer for plant immunity assays, so the Amicon ultracentrifugal polypropylene Ultracel membrane 10KMWCO was used to exchange buffer and concentrate the protein. (b) Ruby staining of a polyacrylamide gel showing the recombinant RcCDI1 protein from *P. pastoris* CS at different stages of the purification process. Total protein corresponds to the initial input (cell-free YPD containing RcCDI1), that is then incubated for 1hr in an Anti-V5 affinity gel, and subsequently poured into a spin column. Unbound sample corresponds to the YPD running through the column. Column is then washed with TBS (wash sample). RcCDI1-V5 was eluted in 8 elution steps using the V5 peptide. Samples from the 2nd, 6th and 8th elution steps are shown in the gel. RCDI1 protein from the combination of the 8 elution steps was purified and concentrated in HNT buffer (Final prep sample). Loading volumes for all samples were 9µL with exception of final prep where the loading volume corresponded to 2 µL. Expected size for RcCDI1 is 23 kDa, observed size in the gel may be explained by the presence of glycosylation.

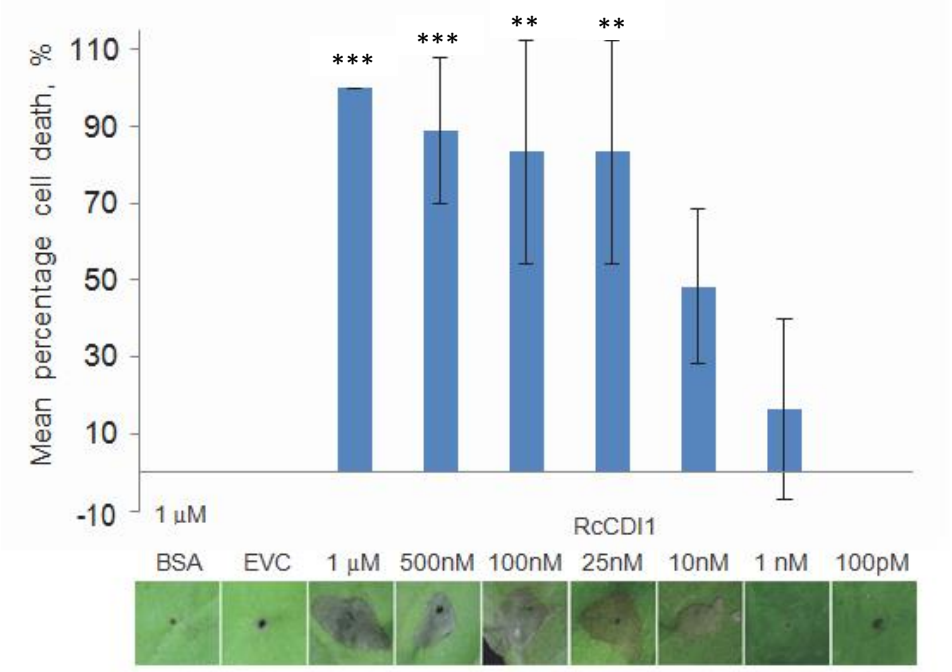

Figure 3.12 *R. commune* protein RcCDI1 at nanomolar concentrations induces cell death in *N. benthamiana* leaves. The percentage of infiltration sites developing a clear cell death in *N. benthamiana* leaves at two days post infiltration (dpi) with purified RcCDI1-V5 protein (100 pM to 1 µM). EV control (*P. pastoris* culture supernatant (CS) from an empty vector control strain expressing V5, purified in the same way as RcCDI1-V5) is the CS control. Photographs of typical infiltration zones are presented in the panel beneath. Experiments were repeated at least two times, each with three to five plants, error bars indicate ± SD. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

To examine the host response specificity to RcCDI1, *P. pastoris* CS containing RcCDI1 or purified RcCDI1 (100 nM for potato, bean and spinach) was infiltrated into leaves of various plant species. RcCDI1-V5 induced very strong localized cell death in solanaceous species, including *N. sylvestris*, tomato (*S. lycopersicum*) cv Moneymaker and potato (*S. tuberosum*) cv Desiree (Figure 3.13a-c), but not in Arabidopsis (*A. thaliana*) wild type Columbia (Col-0), bean (*Vicia faba*) cv Sutton Dwarf, spinach (*Spinacia oleracea*) cv Amazon or monocots, including barley (*H. vulgare*) cv Optic, wheat (*Triticum aestivum*) cv Tybalt, rye (*Secale cereale*), or maize (*Zea mays*) cv Golden Jubilee (Figure 3.13g-j). While areas of *N. benthamiana* and tomato leaves infiltrated with *P. pastoris* CS containing RcCDI1 showed typical fluorescence suggesting the accumulation of phenolic compounds, no response to RcCDI1 have been observed in barley, wheat, rye and maize (Figure 4.3). Sixty-five different

barley genotypes (Table 3.4) were tested, but none of them developed cell death symptoms in response to RcCDI1. Thus, RcCDI1 was able to induce cell death in solanaceous plants but not in other tested dicot families or monocots.

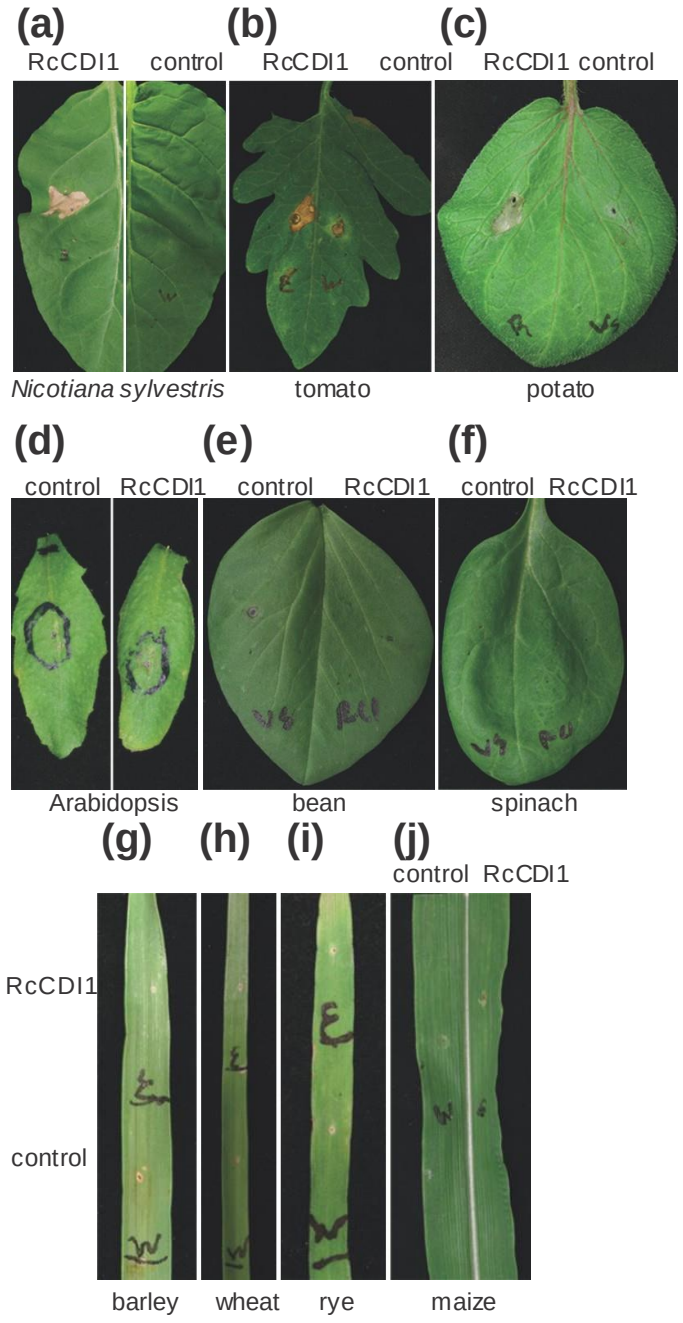

Figure 3.13 RcCDI1 induces cell death in Solanaceae. Representative leaves of (a) *N. sylvestris*, (b) tomato, (c) potato, (d) Arabidopsis, (e) bean, (f) spinach, (g) barley, (h) wheat, (i) rye and (j) maize. Leaves of *N. sylvestris*, tomato, Arabidopsis, barley, wheat, rye and maize were infiltrated with *P. pastoris* culture supernatant (CS) containing RcCDI1-V5 or CS of the original *P. pastoris* strain GS115 expressing V5, while 100 nM of purified RcCDI1-V5 or CS control processed in the same way as RcCDI1-V5 containing culture were used to infiltrate leaves of potato, bean and spinach.

Table 3.4 Barley cultivars and landraces used in the study.

N	Name of cultivar or landrace	*Rhynchosporium* resistance genes
1	Astrix	
2	Atlas	*Rrs2*
3	Atlas 46	*Rrs1, Rrs2*
4	Black Hulless	
5	Doyen	
6	Flagon	
7	Gembloux 456 (CI 8288)	*Rrs15*
8	Nigri Nudum (CI 11549)	*Rrs4*
9	Optic	
10	Pirate	
11	Steudelli	*Rrs3*
12	Triton	*Rrs1*
13	Westminster	
14	JLB_37-002	
15	SLB_03-026	
16	SLB_03-029	
17	SLB_03-054	
18	SLB_05-030	
19	SLB_05-053	
20	SLB_05-097	
21	SLB_09-049	
22	SLB_10-007	
23	SLB_10-009	
24	SLB_12-002	
25	SLB_19-011	
26	SLB_19-094	
27	SLB_22-004	
28	SLB_22-012	
29	SLB_22-013	
30	SLB_22-065	
31	SLB_22-066	

32	SLB_30-010	
33	SLB_30-014	
34	SLB_30-048	
35	SLB_32-014	
36	SLB_32-020	
37	SLB_34-007	
38	SLB_34-018	
39	SLB_34-030	
40	SLB_34-063	
41	SLB_34-065	
42	SLB_34-074	
43	SLB_34-076	
44	SLB_40-038	
45	SLB_40-089	
46	SLB_42-003	
47	SLB_42-008	
48	SLB_42-015	
49	SLB_49-036	
50	SLB_49-048	
51	SLB_58-012	
52	SLB_58-021	
53	SLB_66-023	
54	SLB_66-024	
55	SLB_66-058	
56	SLB_67-007	
57	SLB_67-008	
58	SLB_67-015	
59	SRUC_10.11	
60	SRUC_10.14	
61	SRUC_10.21	
62	SRUC_10.25	
63	SRUC_10.36	
64	SRUC_7.26	
65	SRUC_8.45	

3.3 Discussion

Sequencing of RNA from barley leaves at an early stage of colonisation by *R. commune* identified RcCDI1, a small secreted protein containing four cysteine residues. All four cysteines are conserved in RcCDI1 homologues suggesting formation of two intramolecular disulphide bonds, which may be essential for protein folding and survival in the hostile environment of the plant leaf apoplast.Unfortunately, it does not contain any previously characterised protein domains which might provide clues about its possible function. To characterise RcCDI1, we aimed to overexpress it in a range of barley genotypes, including lines previously shown to contain resistance genes to *R. commune*. Unexpectedly, while producing BSMV VOX inoculum for barley infection, we found that *N. benthamiana* leaves responded to RcCDI1 expression with a strong cell death. Our finding that not only RcCDI1 but also its homologues from other Ascomycetes, including hemi-biotrophic pathogens of cereals: *Z. tritici, M. oryzae*; necrotrophic pathogens of dycots, including Solanaceae: *B. cinerea, S. sclerotiorum,* as well as a saprophyte, *N. crassa*, could trigger cell death in *N. benthamiana* suggested that a widely conserved protein family may be detected by the plant.

All 32 isolates of *R. commune* tested were shown to contain *RcCDI1*, with 6 SNPs identified between isolates. Only 2 of these SNPs led to nonsynonymous substitutions suggesting that purifying selection has acted on this gene to preserve its structure and function. These amino acid changes did not affect RcCDI1 recognition as all three versions of RcCDI1 induced strong cell death in *N. benthamiana* leaves. Cell death induction in *N. benthamiana* required RcCDI1 to be present in the apoplast suggesting that RcCDI1 detection may be mediated by a cell surface pattern recognition receptor.

While chitin remains the best characterised fungal PAMP, several proteinaceous PAMPs have been characterised. In addition to fungal PGs, other cell wall degrading enzymes have been shown to act as PAMPs (Bailey *et al.*, 1990; Ron & Avni, 2004; Nurnberger *et al.*, 2004). The recently identified glycoside hydrolase 12 (GH12) protein, XEG1 is produced by

oomycetes, fungi and bacteria (Ma *et al.*, 2015). Like CDI1, cerato-platanin located in the cell walls of ascospores, hyphae, and conidia is specific to Ascomycetes (Pazzagli *et al.*, 2006; Yang *et al.*, 2009).

RcCDI1 produced by *P. pastoris* triggered cell death in *N. benthamiana*, *N. sylvestris*, tomato and potato, but not in any tested non-solanaceous species of dicots or in monocots, including the host plant barley. Although RcCDI1 did not trigger cell death in cereal species tested we cannot exclude that it does not cause other responses such as ROS or ethylene that are typical for many PAMPs. Many other PAMPs are detected by a restricted range of plants, suggesting that the PRRs recognising them have evolved recently. These include bacterial CSPs, only recognized by Solanaceae, and EF-Tu, recognized by Brassicaceae but not Solanaceae (Felix & Boller, 2003; Kunze *et al.*, 2004). Furthermore, a shortened version of flg22, flg15, is still fully active in tomato but not in Arabidopsis or *N. benthamiana* (Robatzek *et al.*, 2007).

Unlike effectors which are race-specific, PAMPs are highly conserved within a class of microbes. This suggests that transfer of PRRs recognising specific PAMPs to different plant species has tremendous potential to deliver durable resistance against diverse pathogens (Dodds & Rathjen, 2010). For example, transfer of the *A. thaliana* EF-Tu PRR receptor (EFR) only present in the Brassicaceae, into tomato provided good levels of resistance against various bacterial pathogens (Lacombe *et al.*, 2010). Verticillium resistance gene *Ve1*, encoding a RLP-type cell surface receptor also remained fully functional after interfamily transfer from tomato to Arabidopsis making it resistant to race 1 but not to race 2 strains of *V. dahliae* and *V. albo-atrum* (Fradin *et al.*, 2011). Importantly, the resistance signalling was shown to be conserved between tomato and Arabidopsis (Fradin *et al.*, 2011). Thus, identification of the *N. benthamiana* receptor involved in RcCDI1 recognition may provide a valuable resource for engineering nonhost resistance in monocots against a range of economically important pathogens including *Z. tritici* and *M. oryzae*. However, in some cases,

such interspecies gene transfer may lead to undesirable effects on plant growth and development (Bouwmeester *et al.*, 2014).

While potentially providing nonhost resistance against many Ascomycetes, the putative receptor recognising RcCDI1 and its homologues may be exploited as a susceptibility factor by some necrotrophic pathogens, such as *S. sclerotiorum* and *B. cinerea*, capable of infecting solanaceous plants. Previously *B. cinerea* has been shown to exploit the SGT1-mediated cell death pathway to initiate its necrotrophic life style (El Oirdi & Bouarab, 2007). Another necrotroph, *Fusarium culmorum*, has been shown to require AtSGT1b to cause full disease symptoms associated with cell death and tissue dehydration (Cuzick *et al.*, 2009).

In order to confirm an essential role of RcCDI1 for *R. commune*, a gene knockout generation has been attempted. Deletion cassettes for *RcCDI1* were recovered by split marker and transformed into *R. commune* germinated conidia by electroporation, which has been a widely used method to transform filamentous fungi (Chakraborty & Kapoor, 1990). We aimed at obtaining *RcCDI1 R. commune* knockout using the homologous recombination repair pathway (Bhadauria *et al.*, 2009).

After transformation, *R. commune* colonies were growing on selective medium. Amplification of full length hygromycin gene has been achieved for most of the transformants. Unfortunately no amplification was obtained using specific primers to check the correct integration of the selection marker gene (hygromycin) in the right place in the genome replacing the target gene. These results suggest that the transformation procedure worked but *RcCDI1* gene knockout was not achieved. Possible explanation for this result is that, as mentioned previously, homologous recombination rate in a wide range of filamentous fungi, and *R. commune* in particular, is very low due to NHEJ predominance; opposite to *S. cerevisae*, where homologous recombination is highly efficient. Thus, the lack of an available *R. commune* NHEJ deficient strain (for example a Ku70 deletion strain) with increased homologous recombination rate could have hampered successful gene knockout (Carvalho *et*

al., 2010). Other considered reason is that it has been shown that the minimum length of the homologous sequences should be 30 bp for the recombination to occur in yeast (Hua *et al.*, 1997), but in filamentous fungi it requires a minimum of 600 bp for each of the flanking region to increase the chances of the recombination process happening (Kooistra *et al.*, 2004). In this case each of the flanking regions had a minimum length of 1 Kb, so is not considered as a main reason for the unsuccessful result. Another possibility is that RcCDI1 gene knockout may be lethal to *R. commune*. In this case the use of RNAi is recommended in order to decrease the expression of the gene rather than disrupt or delete the gene (Weld *et al.*, 2006). In conclusion, to further aid with RcCDI1 characterisation we tried to obtain a gene knockout strain but our aim was not accomplished. The actual cause for this remains unknown, so more effort needs to be put into the *RcCDI1* gene knockout strategy in order to make it work effectively or search for the most suitable method to evaluate the function of RcCDI1.

The identification of the Ascomycete PAMP RcCDI1, recognised by solanaceous species but not monocots is an important step toward identifying new resistance mechanisms that may be transferred between plant families. The ultimate goal would be to engineer globally important cereal crop plants wheat, rice and barley with durable resistance against Ascomycetes while avoiding adverse effects on plant growth and development.

Chapter 4. PTI components involved in the RcCDI1 recognition

4.1 Introduction

Plant diseases are a major threat to agriculture worldwide, causing serious reductions in crop yields and quality (Dixon, 2012). Pathogen epidemics are regulated by several factors such as host susceptibility, virulence of the pathogen and suitable climatic conditions (Agrios, 2005), and under some of these conditions, the pathogen is often defined as a very variable organism able to overcome resistance and chemical control (Strange & Scott, 2005). Amongst these destructive pathogens, ascomycetes represent one major group containing the fungal pathogen *R. commune*, indicated as one of the major threats to barley crop production worldwide, causing the disease known as leaf blotch and leading to significant yield losses (Shipton *et al*., 1974). Given its important role, an improved understanding of the biology of this pathogen is required when aiming for informed strategies for controlling *R. commune* in the field.

The evolution of plants and their pathogens was driven by an arms race usually described in the zigzag model (Jones & Dangl, 2006). One of the components of this evolutionary model involves the perception of PAMPs or MAMPs by PRRs located on the plant cell surface triggering the first layer of defence referred to as PTI (Jones & Dangl, 2006; Boller & Felix, 2009; Muthamilarasan & Prasad, 2013).

The perception of PAMPs by plants leads to the activation of defense responses, including changes in ion fluxes in the plant plasma membrane; increase in cytosolic Ca^{2+} levels; reactive oxygen species (ROS) production; callose deposition; mitogen-activated protein kinases (MAPK) cascade activation; induction of hormones such as ethylene, JA and SA; and activation of WRKY transcription factors leading to subsequent transcription of defence related genes (Felix *et al.*, 1999; Gómez-Gómez *et al.*, 1999; Grant *et al.*, 2000; Asai *et al.*, 2002; Gómez-Gómez & Boller, 2002; Benschop *et al.*, 2007; Boller & Felix, 2009; Nakagami *et al.*, 2010; Bigeard *et al.*, 2015).

RcCDI1 is a novel protein from the fungal pathogen *R. commune* suggested to act as a PAMP based on the results described in the previous chapter. It was shown that RcCDI1 homologues from species such as *N. crassa, Z. tritici, M. oryzae, B. cinerea* and *S. sclerotiorum* induced cell death in solanaceous plants once present in the leaf apoplast. However, the mechanism by which this protein induces cell death remains unknown.

Most PRRs, including those recognising bacterial flg22, elf18, harpin, LPS, CSPs, oomycete elicitor PiINF1 and NLPs in model plants *A. thaliana* and *N. benthamiana*, interact with the LRR-RLK BAK1 to initiate PTI (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Shan *et al.*, 2008; Albert *et al.*, 2015) (Figure 4.1). One exception is CERK1, which does not require BAK1 (Miya *et al.*, 2007; Wan *et al.*, 2008).

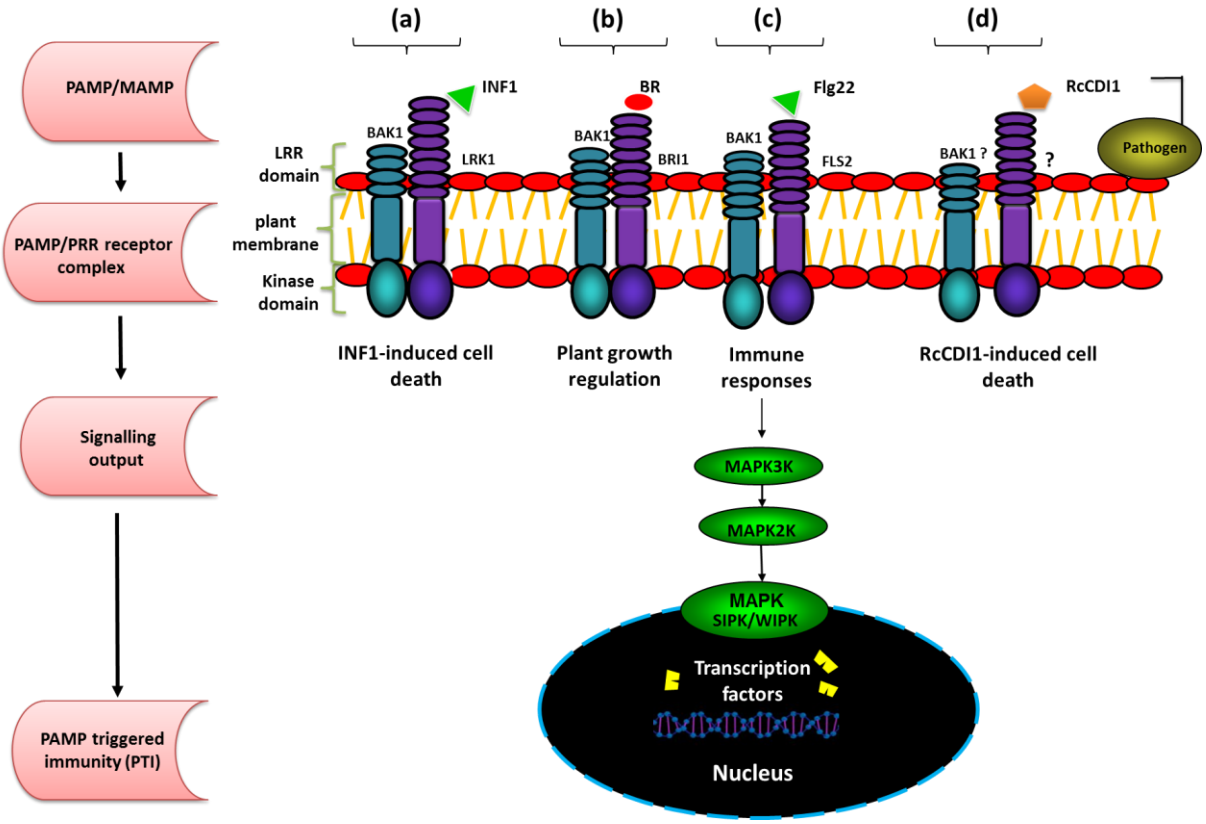

Figure 4.1 Perception of PAMPs/MAMPs by PRRs (Based on figure from Ma *et al.*, 2016). PAMPs are perceived by PRRs located on the cell surface in association with their co-receptor BAK1. This PAMP/PRR receptor complex leads to the activation of different signalling pathways including MAPK cascades for the induction of plant defence responses. (a) *P. infestans* elicitin INF1 is recognised by the RLK receptor protein LRK1, mediating INF1-induced cell death (b) BRs are recognised by the RLK receptor protein BRI1, activating signalling involved in BR-mediated plant growth. (c) Flg22 perception by RLK receptor protein FLS2 activating FLS2-mediated immune signalling. (d) RcCDI1 perception by a still unidentified plant surface receptor mediating RCDI1-induced cell death via the activation of yet unknown signalling pathways.

Another LRR-RLK SOBIR1 was shown to be essential for triggering defence responses by certain LRR-RLPs acting as immune receptors (Gao *et al.*, 2009). While BAK1 has a general regulatory role in plasma membrane-associated receptor complexes comprising LRR-RLPs and/or LRR-RLKs, SOBIR1 is specifically required for the function of receptor complexes containing LRR-RLPs (Liebrand *et al.*, 2014).

SGT1, a homologue of the yeast ubiquitin ligase-associated protein, plays an important role in plant ETI mediated by multiple types of R proteins recognising both intracellular and extracellular pathogen-derived elicitors (Peart *et al.*, 2002; Austin, 2002; Azevedo, 2002; Tor,

2002). Furthermore, SGT1 is essential for *N. benthamiana* nonhost resistance against various bacterial pathogens as well as PTI response to the *P. infestans* PAMP PiINF1 (Peart *et al.*, 2002).

Another ubiquitination-associated protein, U-box E3 ligase CMPG1 is involved in PTI and ETI responses. CMPG1 is required for cell death triggered at the host plasma membrane by combinations of *C. fulvum* effectors Avr4 or Avr9 with cognate tomato resistance proteins Cf4 or Cf9, respectively, as well as *P. syringae* effector AvrPto and resistance protein Pto, or the oomycete PAMPs PiINF1 and CBEL (Gonzalez-Lamothe, 2006; Bos *et al.*, 2010; Gilroy *et al.*, 2011). *P. infestans* RxLR effector AVR3a^{KI} interacts with CMPG1, stabilizing it and suppressing cell death triggered by a variety of effectors and PAMPs (Bos *et al.*, 2010; Gilroy *et al.*, 2011).

Other key players in both PRR and NB-LRR–mediated immunity are mitogen-activated protein kinases, MAPK (Martin *et al.*, 2003; Boller & Felix, 2009; Segonzac *et al.*, 2011). MAPKKKε has been identified as important for resistance in tomato to certain strains of plant pathogenic bacteria and for mediating responses downstream of certain effector/R protein combinations in *N. benthamiana* (Melech-Bonfil & Sessa, 2010). *P. infestans* RxLR effector PexRD2 suppresses MAPKKKε-mediated cell death, elicited by effector/R protein pairs, including Cf4/Avr4 and Pto/AvrPto (King *et al.*, 2014).

PAMP perception leads to the transcriptional activation of defence-related genes (Asai *et al.*, 2002; McLellan *et al.*, 2013). For this reason, we were interested to test if RcCDI1 triggered the transcriptional upregulation of genes involved in PTI signaling pathways (*WRKY7*, *WRKY8*, *ACRE31*, *PTI5*) in *N. benthamiana.*

WRKY transcription factors belong to a very large family of transcription factors (Eulgem *et al.*, 2000). They play important roles as regulators of several plant biological processes, especially those associated with plant immunity. *WRKY7/8* genes were shown to be upregulated in *N. benthamiana* after flg22 treatment (Adachi *et al.*, 2015).

There are several similarities in the defence responses activated against fungal and bacterial pathogens upon detection of PAMPs. For instance, both bacterial and fungal PAMPs induce the expression of defence genes such as the bacterial Flagellin Rapidly Elicited (*FLARE*) and the fungal Avr9/Cf-9 Rapidly Elicited (*ACRE*) (Rowland *et al.*, 2005). *ACRE* genes encoding protein kinases like Avr9/Cf-9 induced kinase 1 (ACIK1) were highly expressed in tomato and tobacco after Avr9 elicitation (Rowland *et al.*, 2005). Pathogenesis-related genes transcriptional activator *(PTI5)* is an ethylene-response factor (ERF) transcription factor previously described as a Pto interactor based on an yeast-two hybrid screen assay (Zhou *et al.*, 1997). *Pto* is a resistance gene encoding a serine/threonine kinase that mediates tomato resistance to *P. syringae* pv. *tomato* strain DC3000 expressing the avirulence gene avrPto (Martin *et al.*, 1993).

In the previous chapter, RcCDI1 was shown to induce cell death in solanaceous species but not in monocots, including its host barley. The absence of cell death does not exclude the possibility of other defence responses, as there are many different types of responses in addition to the activation of cell death, as mentioned previously with the induction of defence-related genes during PTI and ETI signalling (De Vleesschauwer *et al.*, 2014).

For this reason, *PR1* and Allene Oxide synthase (*AOS*) (as SA and JA-responsive marker genes respectively) up-regulation upon RcDI1 treatment was tested in barley. PR1 is a very well-known marker for *SA*-mediated defence responses (Ward *et al.*, 1991). Previous studies have shown that plant systemic defence responses, as SA accumulation and expression of SA related genes like *PR1*, are activated after flg22 treatment in *A. thaliana* (Mishina & Zeier, 2007). In addition, AOS is the first enzyme involved in the pathway for JA biosynthesis (Mueller, 1997). In Arabidopsis, the up regulation of AOS was induced by wounding (Kubigsteltig *et al.*, 1999). Work done by (Mei *et al.*, 2006) showed that transgenic rice lines overexpressing AOS gene increased the activation of JA biosynthesis in response to *M. oryzae*, the rice blast fungus.

In summary, this work provides evidence that RcCDI1 cell death is BAK1, SOBIR1 and SGT1 dependent and NbCMPG1 and NbMAPKKKε independent. Besides, RcCDI1 was shown to induce transcription of the PTI marker genes *WRKY7*, *WRKY8*, *ACRE31*, *PTI5* in *N. benthamiana*. Silencing of *NbBAK1* was shown to prevent upregulation of these PTI marker genes by RcCDI1. This work improves our understanding of the resistance mechanisms triggered by RcCDI1 in *N. benthamiana* that could be crucial for future efforts aimed at searching for barley resistance against *R. commune*.

4.2 Results

4.2.1 Tobacco BAK1/SERK3, SOBIR1 and SGT1 are required for RcCDI1-triggered cell death in *N. benthamiana*

The RLKs BAK1/SERK3 and SOBIR1 were shown to interact with various PRRs to facilitate intracellular signalling (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Liebrand *et al.*, 2014; Albert *et al.*, 2015). To investigate whether *NbBAK1* and *NbSOBIR1* are required for the induction of cell death mediated by RcCDI1, VIGS constructs were used to independently target *NbBAK1* and *NbSOBIR1* expression in *N. benthamiana*. Two weeks after inoculation with TRV VIGS constructs, the levels of expression of BAK1 and SOBIR1 were lower in *NbBAK1* and *NbSOBIR1*-silenced plants than in TRV:EV plants (88% reduction for BAK1 and 80% reduction for SOBIR1). (Figure 4.2c,g). Besides, the plants were showing the expected phenotype consisting of a semi-dwarf stature and distorted leaves (Heese *et al.*, 2007) and both sets of plants were infiltrated with *P. pastoris* CS containing RcCDI1-V5 or PiINF1-V5. While both RcCDI1-V5 and PiINF1-V5 triggered cell death in *N. benthamiana* inoculated with TRV:EV (Figure 4.2a,b,e,f), as anticipated, silencing of *NbBAK1* or *NbSOBIR1* led to significant reduction in the proportion of PiINF1 infiltration sites showing cell death (Figure 4.2b,f). Similar to PiINF1, RcCDI1-triggered cell death was also affected in the majority of *NbBAK1*- or *NbSOBIR1*-silenced plants, with the proportion of infiltration

sites showing cell death reduced from 94 % to 28 % and from 73.3 % to 22 % respectively (Figure 4.2a,b,e,f).

To investigate the involvement of another common component of plant innate immunity in *N. benthamiana* responses to RcCDI1, VIGS constructs were used to knock-down expression of *NbSGT1*. Two weeks after inoculation with TRV constructs, the levels of expression of SGT1 were lower in *NbSGT1*-silenced plants than in TRV:EV plants (95% reduction) (Figure 4.2j). Besides, the plants were showing the expected phenotype consisting of a semi-dwarf stature and more branched (Peart *et al.*, 2002), were infiltrated with *P. pastoris* CS containing RcCDI1-V5 or the V5 tag (Figure 4.2i). Co-infiltration with *A. tumefaciens* cultures expressing the *P. infestans* effector protein PiAvr3a and the cognate cytoplasmic potato resistance protein R3a was used as a positive control for inducing SGT1-dependent cell death in *N. benthamiana* leaves (Bos *et al.*, 2006). While both RcCDI1-V5 and a R3a/Avr3a pair triggered cell death in *N. benthamiana* inoculated with TRV:EV (Figure 4.2i), silencing of *NbSGT1* led to significant reduction in the proportion of R3a/Avr3a infiltration sites showing cell death (Figure 4.2i). RcCDI1-triggered cell death was also affected in the majority of these plants with the proportion of infiltration sites showing cell death reduced from 81 % in the TRV:EV infected plants to 46 % in the *NbSGT1*-silenced plants (Figure 4.2i). These results suggest that NbBAK1, NbSOBIR1 and NbSGT1 play a role in RcCDI1-triggered cell death in *N. benthamiana*. Presence of RcCDI1-V5 or PiINF1-V5 were confirmed in control (TRV:EV), *NbBAK1* (TRV:*BAK1*), *NbSOBIR1* (TRV:*SOBIR1*) and *NbSGT1* (TRV:*SGT1*) silenced plants (Figure 4.2d,h,k).

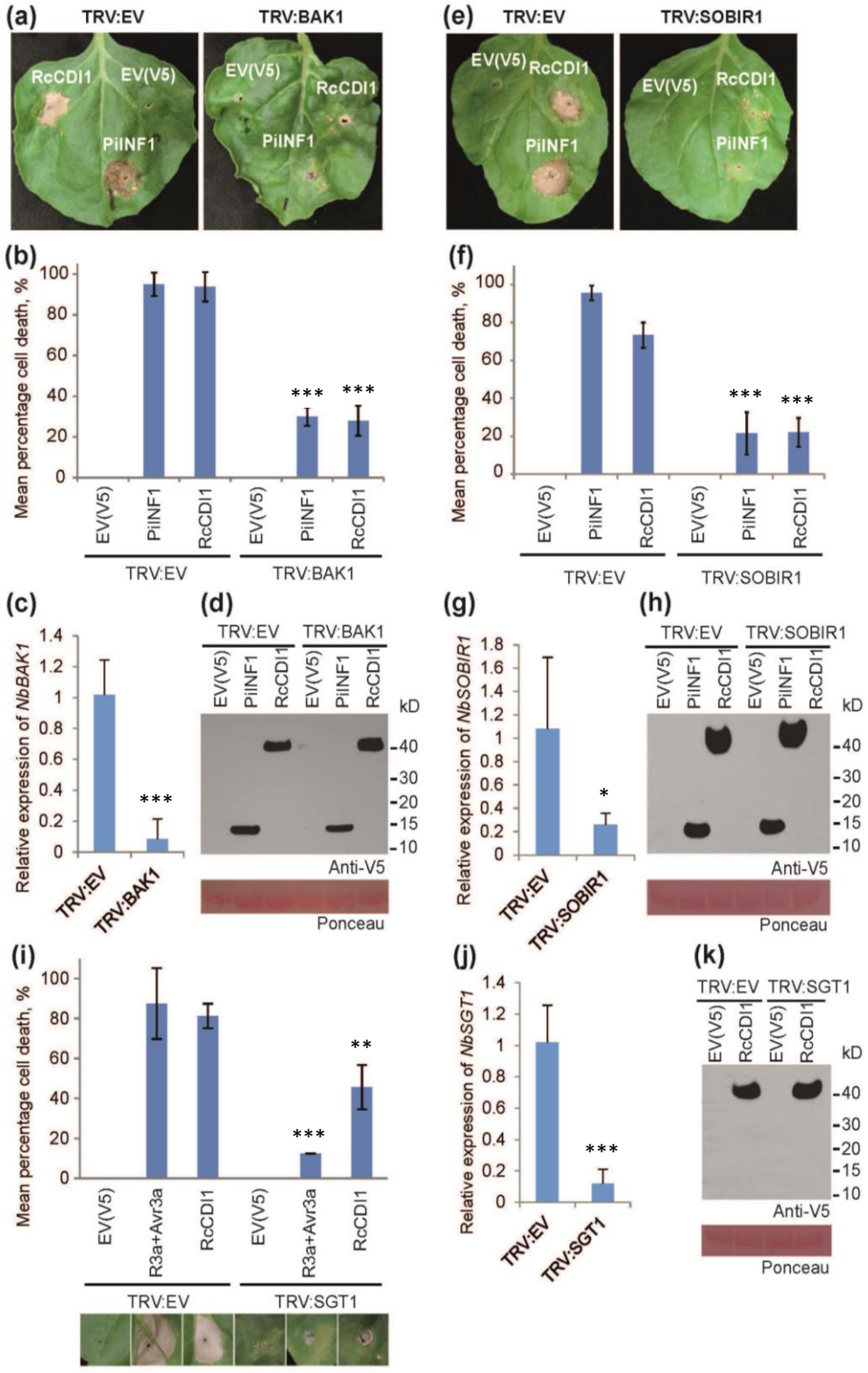

Figure 4.2 Cell death triggered by RcCDI1 requires NbBAK1, NbSOBIR1 and NbSGT1. (a), (e) *N. benthamiana* plants infiltrated with *A. tumefacien*s cultures carrying mixtures of TRV RNA1 and TRV RNA2 vectors (TRV:EV or TRV:*NbBAK1* or TRV:*NbSOBIR1*) showed the expected phenotype for each of the constructs two weeks after inoculation. The leaves of the plants inoculated with TRV:EV and *NbBAK1*-silenced or *NbSOBIR1*-silenced leaves were infiltrated with *P. pastoris* culture supernatant (CS) containing EV (V5), PiINF1-

V5 or RcCDI1-V5. Leaves were photographed seven days later. Photographs of representative plant responses are shown. (b), (f) The percentage of infiltration sites developing cell death in *N. benthamiana* leaves at five dpi with *P. pastoris* CS containing EV (V5), PiINF1-V5 or RcCDI1-V5. The experiment was performed four times with at least eight plants for each TRV construct. Error bars indicate ± SD. (c), (g), (j) *NbBAK1, NbSOBIR1* and *NbSGT1* expression levels after VIGS treatment determined by qRT-PCR analysis. *N. benthamiana tubulin* was used as an endogenous control. Means and standard deviations (SD) from six biological replicates are shown. (d), (h), (k) Immunoblot analysis of proteins from *N. benthamiana* leaves one h post infiltration with *P. pastoris* CS containing EV (V5), PiINF1-V5 or RcCDI1-V5. (i) The percentage of infiltration sites developing cell death in the leaves of *N. benthamiana* plants infiltrated with *A. tumefaciens* cultures carrying mixtures of TRV RNA1 and TRV RNA2 vectors (TRV:EV or TRV:*NbSGT1*) at five dpi with *P. pastoris* CS containing EV (V5) or RcCDI1-V5. Co-infiltration with *A. tumefaciens* cultures expressing R3a and Avr3a was used as a positive control for inducing NbSGT1-dependent cell death in *N. benthamiana* leaves. *NbSGT1*-silenced plants showed the expected phenotype two weeks after inoculation. The experiment was performed three times with at least eight plants for each TRV construct. Photographs of typical infiltration zones are presented in the panel beneath. Error bars indicate ± SD. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

4.2.2 RcCDI1 induces the expression of PTI marker genes in *N. benthamiana*

The PTI marker genes *NbPTI5, NbACRE31, NbWRKY7* and *NbWRKY8* were shown to be induced by flg22 (McLellan *et al.*, 2013). To further characterise *N. benthamiana* responses to RcCDI1 the transcription levels of these PTI marker genes were analysed using qRT-PCR in *N. benthamiana* leaves. Despite the fact that the PiINF1 elicitin from *P. infestans* has been characterised as a PAMP (Bos *et al.*, 2006; Heese *et al.*, 2007; Hann & Rathjen, 2007; Chaparro-Garcia *et al.*, 2011), so far only *NbACRE31* was shown to be induced by PiINF1 (Chaparro-Garcia *et al.*, 2015). Nevertheless, purified PiINF1-V5 from *P. pastoris* CS was used as a protein reference in these assays. A time course was performed for choosing the best time to collect samples for the later qPCR analysis of the expression of the PTI marker genes after protein infiltration. Samples were collected at 0, 0.5, 1, 3 and 6 hours after infiltration with *P. pastoris* CS containing RcCDI1-V5 compared to the EV (V5) (Figure 4.3a). The expression of the genes *WRKY7* and *WRKY8* was monitored, and they were specifically up-regulated by RcCDI1-V5 early at 1 h post infiltration, which prompted us to choose it as the best time point to test the expression of all four marker genes in evaluation after RcCDI1-V5 and EV (V5) infiltration (Figure 4.3a). To further characterise *N. benthamiana* responses to

RcCDI1 the transcription levels of these PTI marker genes were analysed using qRT-PCR in *N. benthamiana* leaves 1 hour post infiltration with *P. pastoris* CS containing RcCDI1-V5 or EV (V5), and 1 hour (*NbPTI5*, *NbACRE31*, *NbWRKY7*) and 30 min (*NbWRKY8*) post infiltration with purified PiINF1-V5 or V5 peptide. The marker genes *NbPTI5*, *NbACRE31*, *NbWRKY7* and *NbWRKY8*, were up-regulated by *P. pastoris* CS containing RcCDI1-V5 compared to the EV (V5) (Figure 4.3b), further confirming it to be a PAMP. Similarly, the same marker genes were up-regulated by purified PiINF1-V5 when compared to V5 peptide (Figure 4.3c). Upregulation of these PTI marker genes was prevented in BAK1-silenced plants (Figure 4.3c,d).

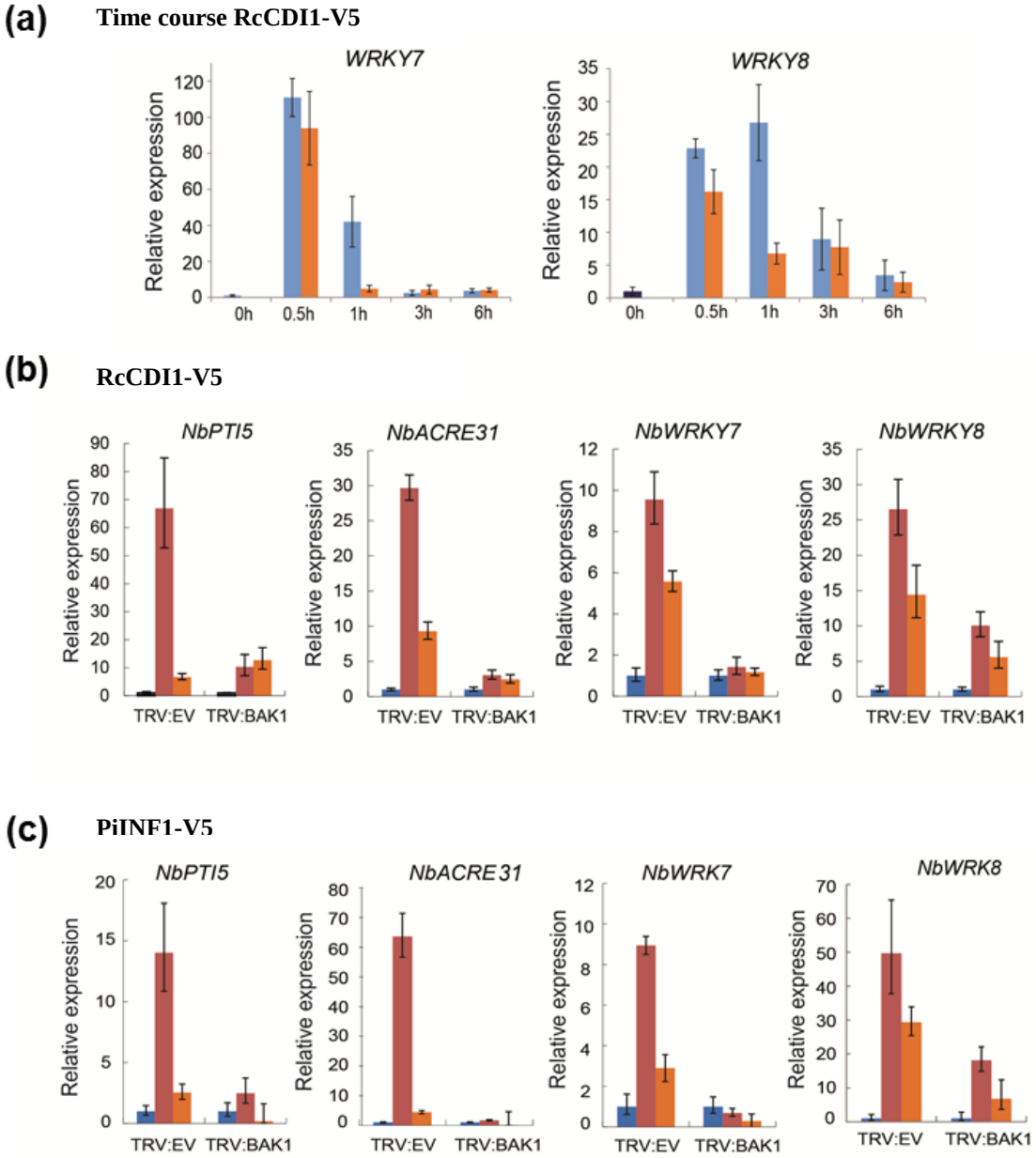

Figure 4.3 Transcriptional upregulation of *Nicotiana benthamiana* PTI marker genes triggered by RcCDI1 and PiINF1 is prevented in BAK1-silenced plants. (a) Relative transcript abundance of *WRKY7* and *WRKY8* genes in *N. benthamiana* plants between 0.5 and 6 h post infiltration with *P. pastoris* CS containing RcCDI1-V5 (light blue) or EV (V5) (orange) compared to pre-infiltration levels (dark blue) assigned the value 1.0. (b) Relative transcript abundance of *NbPTI5*, *NbACRE31*, *NbWRKY7* and *NbWRKY8* in *N. benthamiana* plants inoculated with TRV:EV and *NbBAK1*-silenced leaves 1 hour post infiltration with *P. pastoris* culture supernatant containing RcCDI1-V5 (red) or EV (V5) (orange) compared to pre-infiltration levels (blue) assigned the value 1.0. (c) Relative transcript abundance of *NbPTI5, NbACRE31, NbWRKY7* and *NbWRKY8* in *N. benthamiana* plants inoculated with TRV:EV and *NbBAK1*-silenced leaves 1 hour post infiltration, except for *WRKY8* at 30 min post infiltration with *P. pastoris* culture supernatant containing PiINF1-V5 (red) or V5 peptide (orange) compared to pre-infiltration levels (blue) (assigned the value 1.0). Assays were repeated on three independent occasions, using leaf material from three independent inoculations and generated similar expression profiles. Shown are the representative data from one such experiment.

4.2.3 RcCDI1-triggered cell death in *N. benthamiana* is not suppressed by effectors PiAVR3a and PexRD2

Cell death in plants can be suppressed by pathogen effector proteins (Abramovitch, 2003). One well characterised effector is *P. infestans* PiAvr3a^{KI}, previously shown to suppress CMPG1-dependent cell death in *N. benthamiana* leaves, triggered by a range of pathogen elicitors (Bos *et al.*, 2010; Gilroy *et al.*, 2011). Several positive controls known to induce cell death in *N. benthamiana* leaves, including *P. infestans* elicitin PiINF1 and a combination of *P. infestans* PiAvr3a^{KI} with the corresponding potato resistance protein R3a, as well as negative controls such as an empty binary expression vector, or R3a or PiAvr3a^{KI} alone, were also used for agrobacterium infiltration in this experiment (Figure 4.4a). While, as expected, PiAvr3a^{KI} suppressed cell death triggered by PiINF1 (Bos *et al.*, 2006), it had no effect on cell death triggered by RcCDI1 (Figure 4.4a,b). This provides indirect evidence that NbCMPG1 is likely not required for development of cell death in *N. benthamiana* in response to RcCDI1. The same outcome was observed in response to the co-expression of *S. sclerotiorum* homologue SsCDI1 and PiAvr3a^{KI} in *N. benthamiana*. PiAvr3a^{KI} suppressed cell death triggered by PiINF1 but not by SsCDI1 (Figure 4.5).

A second *P. infestans* RxLR effector, PexRD2, previously shown to suppress MAPKKKε-dependent cell death (King *et al.*, 2014), was co-expressed with RcCDI1. The *C. fulvum* effector AvrCf4 was co-expressed with the cognate tomato resistance protein Cf4 as a positive control as it induces MAPKKKε-dependent cell death in *N. benthamiana* (King *et al.*, 2014). While, as expected, PexRD2 suppressed cell death triggered by co-expression of Avr4/Cf4, it had no effect on cell death triggered by RcCDI1 (Figure 4.4c,d). This suggests that RcCDI1-triggered cell death is independent of *N. benthamiana* MAPKKKε. Importantly, the failure of AVR3aKI or PexRD2 to suppress RcCDI1-triggered cell death demonstrates that this immune response pathway is novel.

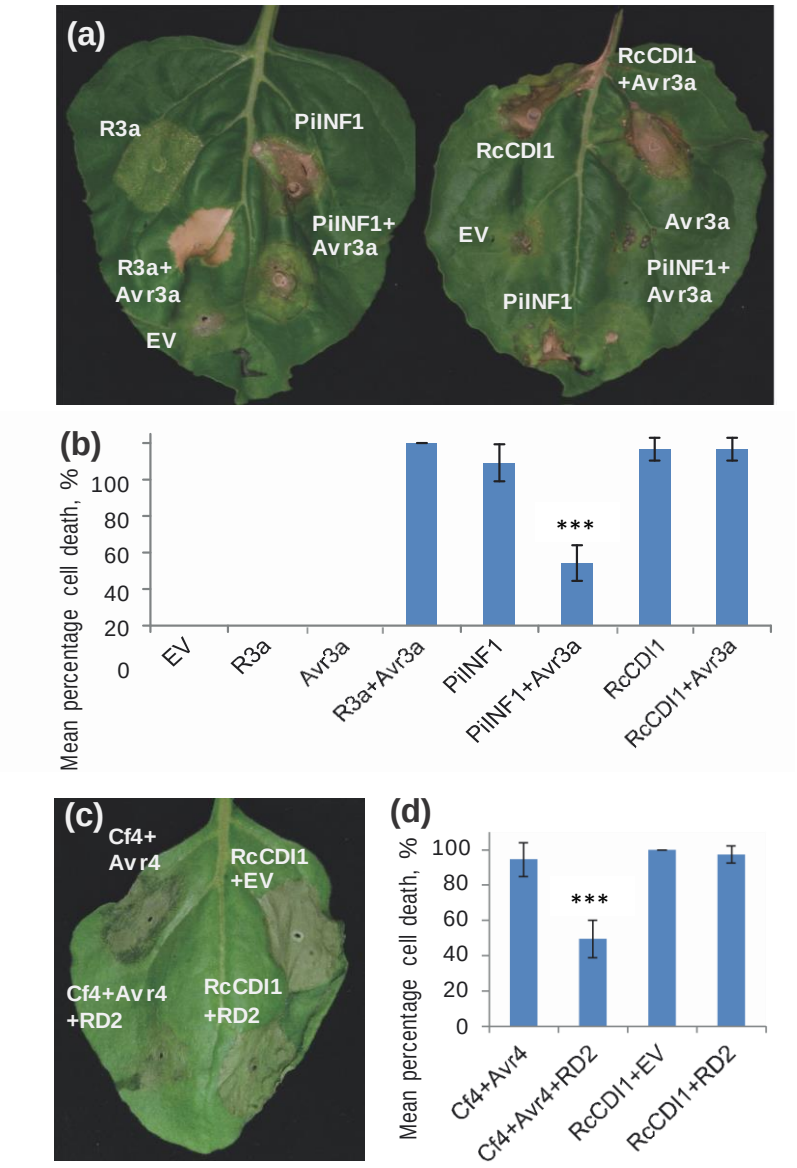

Figure 4.4. CMPG1 and MAPKKKε are not required for RcCDI1-triggered cell death in *Nicotiana benthamiana*. (a) Representative *N. benthamiana* leaf at seven days post co-infiltration (dpi) using constructs encoding the indicated proteins and pGR106-empty vector control (EV). While Avr3a^{KI}, interacting with CMPG1, suppressed cell death triggered by *P. infestans* elicitor PiINF1, it had no effect on cell death induced by *R. commune* RcCDI1. (c) Representative *N. benthamiana* leaves seven dpi using constructs encoding the indicated proteins and pGR106-empty vector control (EV). While PexRD2 suppressed MAPKKKε-dependent cell death triggered by co-expression of Cf4 with Avr4, it had no effect on RcCDI1-induced cell death. (b, d) The percentage of infiltration sites developing cell death in *N. benthamiana* leaves at seven dpi with constructs encoding the indicated proteins. Experiments were repeated at least three times, each with no less than seven plants with three leaves infiltrated per plant, error bars indicate ± SD. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; ***$P \leq 0.001$.

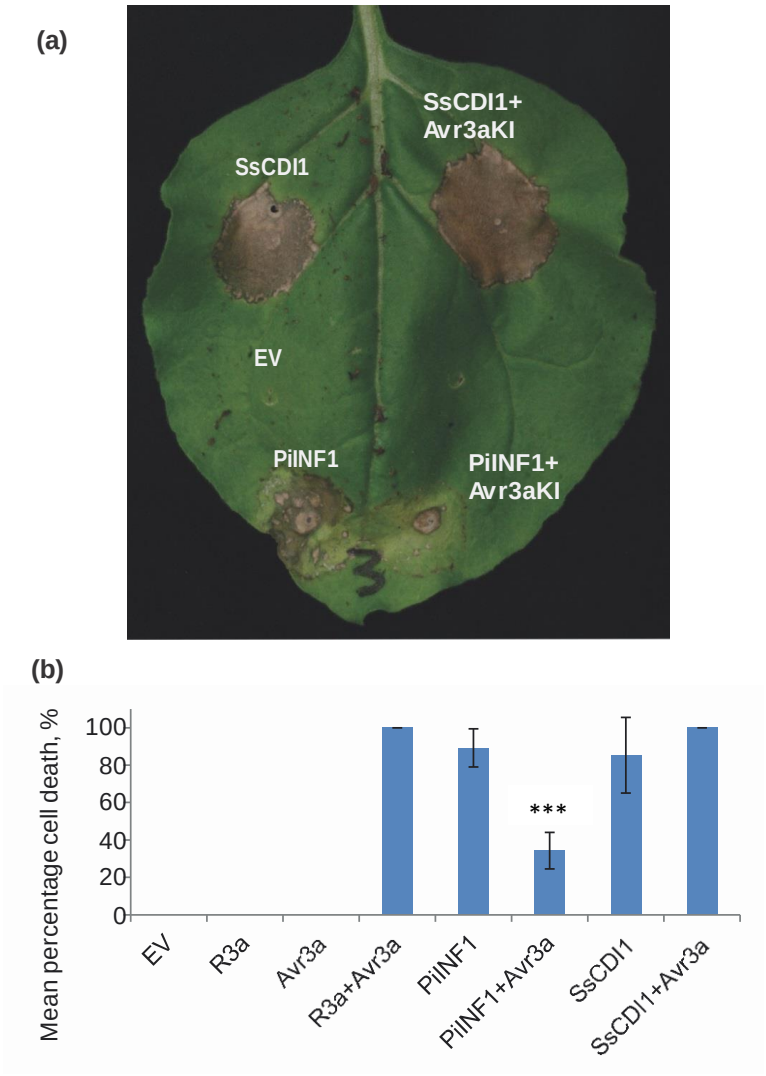

Figure 4.5 CMPG1 is not required for *S. sclerotiorum* CDI1 (SsCDI1)-triggered cell death in *Nicotiana benthamiana*. (a) Representative *N. benthamiana* leaves at 7 days after co-agroinfiltration using constructs encoding the indicated proteins. (b) While Avr3a suppressed cell death triggered by *P. infestans* elicitor PiINF1, it had no effect on cell death induced by SsCDI1. Experiments were repeated at least three times, each with no less than seven plants with three leaves infiltrated per plant, error bars indicate ± SD. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; ***$P \leq 0.001$.

4.2.4 Does RcCDI1 induce the transcription of PTI marker genes in barley?

Previously we showed that infiltration of barley leaves with *P. pastoris* CS containing RcCDI1-V5 did not cause HR (Figure 3.13, 4.6a). As PAMPs can induce PTI transcriptional responses that do not lead to cell death we set up to test the upregulation of SA- and JA-responsive genes in susceptible barley cv Optic. Two marker genes: the SA marker, HvPR1,

and the JA marker, HvAOS, were evaluated in barley leaves 4 hours and 20 min, respectively, post infiltration with *P. pastoris* CS containing RcCDI1-V5 or EV (V5). It has been shown that defence responses of barley to *R. commune* include the induction of PR proteins. NIP1, was shown to elicit the accumulation of *PR1, PR5, PR9* and *PR10* mRNA in barley (Steiner-Lange *et al.*, 2003). NIP1 was not available for this experiment, but NIP2-V5 and NIP3-V5 were used as reference proteins for the induction of *PR1*. Expression of *PR1* was slightly induced by NIP3-V5 compared to water and *P. pastoris* CS containing V5 (Figure 4.6b). Both, *HvPR1* and *HvAOS* marker transcripts were slightly more abundant in barley leaves infiltrated with *P. pastoris* CS containing RcCDI1-V5 (Figure 4.6b). Nevertheless the induction of these genes was not very high, especially for *HvAOS* and these levels are likely to be within normal variation in transcript abundance (Figure 4.6b).This is supported by the difference in *HvAOS* transcript abundance between barley leaves infiltrated with water and *P. pastoris* CS containing V5 (Figure 4.6b). The experiment needs repeating to see whether RcCDI1 has any effect on *HvPR1* and *HvAOS* transcripts.

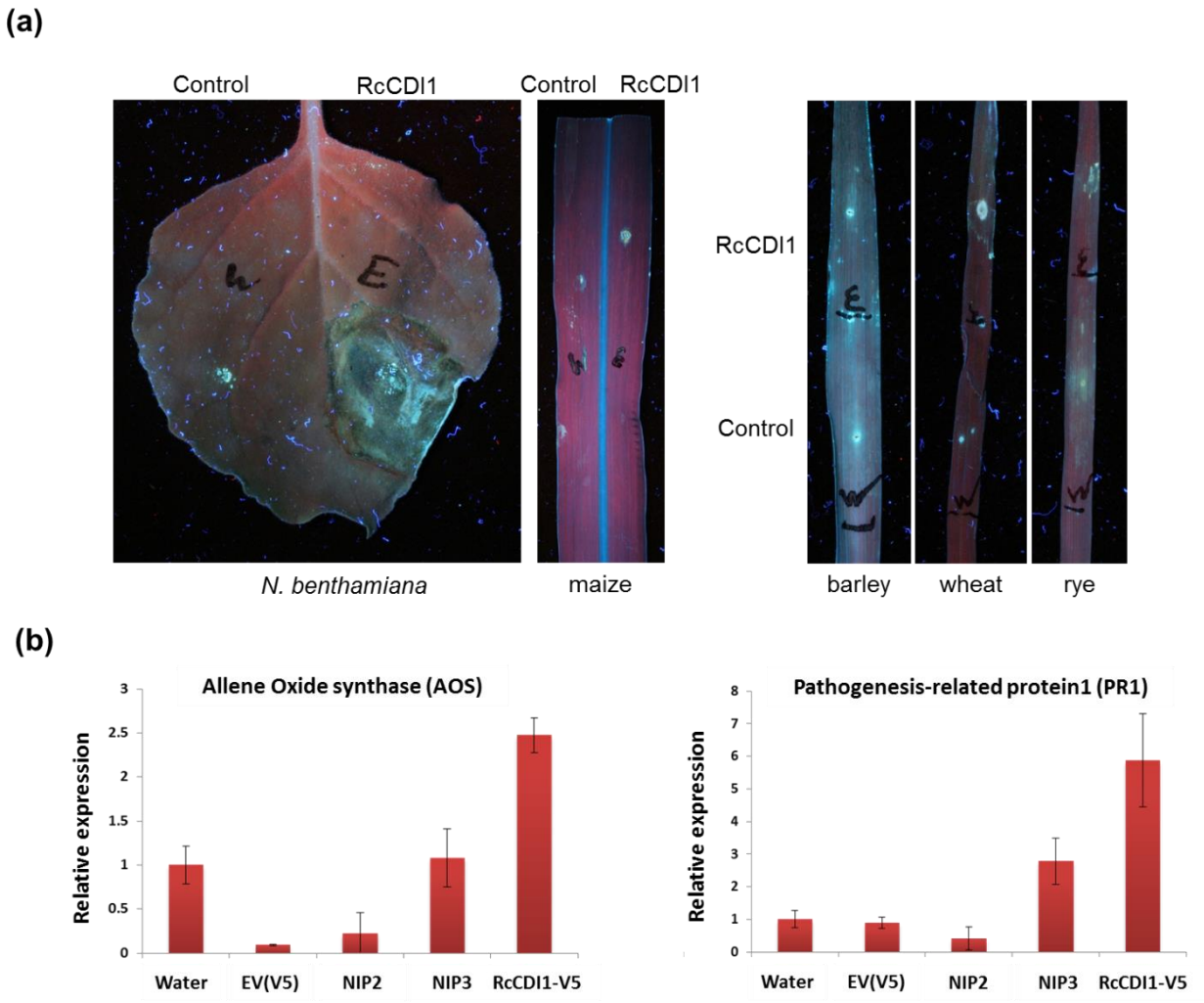

Figure 4.6 Barley PTI marker genes response to RcCDI1. (a) UV-light was used to detect fluorescence due to the accumulation of total phenolic compounds caused by the cell death response of *N. benthamiana* leaves infiltrated with *P. pastoris* CS containing RcCDI1. In contrast, no cell death response was observed in barley, wheat, rye and maize after infiltration, so no fluorescence was detected. (b) Relative transcript abundance of the genes *HvPR1* and *HvAOS* in barley cv Optic 4 h and 20 min post infiltration respectively, with *P. pastoris* culture supernatant containing RcCDI1-V5, EV (V5), NIP2-V5 or NIP3-V5 compared to water infiltration levels assigned the value 1.0. Error bars represent confidence intervals calculated using standard deviations from three technical replicates for each sample within the qRT-PCR assay.

4.2.5 The investigation of other potential pathways involved in RcCDI1 recognition

Previous studies have shown that in tomato, disease resistance to *P. syringae* pv. tomato is led by the interaction of the Pto resistant protein with the avirulence protein AvrPto manifested as HR which restricts the proliferation of the pathogen (Shan *et al.*, 2000). In contrast, it has also been shown that AvrPto is able to suppress immune responses upstream of MAPK cascade upon PAMP perception, targeting the Arabidopsis RLKs FLS2 and EFR and tomato LeFLS2 (Xiang *et al.*, 2008). Xiang *et al.*, (2008) also demonstrated that the FLS2-AvrPto interaction does not require BAK1. Similar to AvrPto, the *P. infestans* RxLR effector, PIGT_13628 (PexRD27) was previously shown to suppress early Flg22-inducible reporter gene activation in tomato and Arabidopsis protoplasts (Zheng *et al.*, 2014). Results published by Zheng *et al.*, (2014) indicate that PIGT_13628 targets the flg22/FLS2 MAP kinase cascade, but do not suppress MAP kinase cascades leading to Cf4- or INF1-mediated PCD. In this order of ideas, we were interested to test if AvrPto and/or PIGT_13628 are able to supress the cell death triggered by RcCDI1 in *N. benthamiana*. Neither AvrPto, or PIGT_13628 had any effect on the cell death triggered in *N. benthamiana* by RcCDI1 (Figure 4.7a,b). These results suggest that RcCDI1-triggered cell death is independent of any of the signalling pathways affected by the avirulence protein AvrPto and the RxLR effector PIGT_13628.

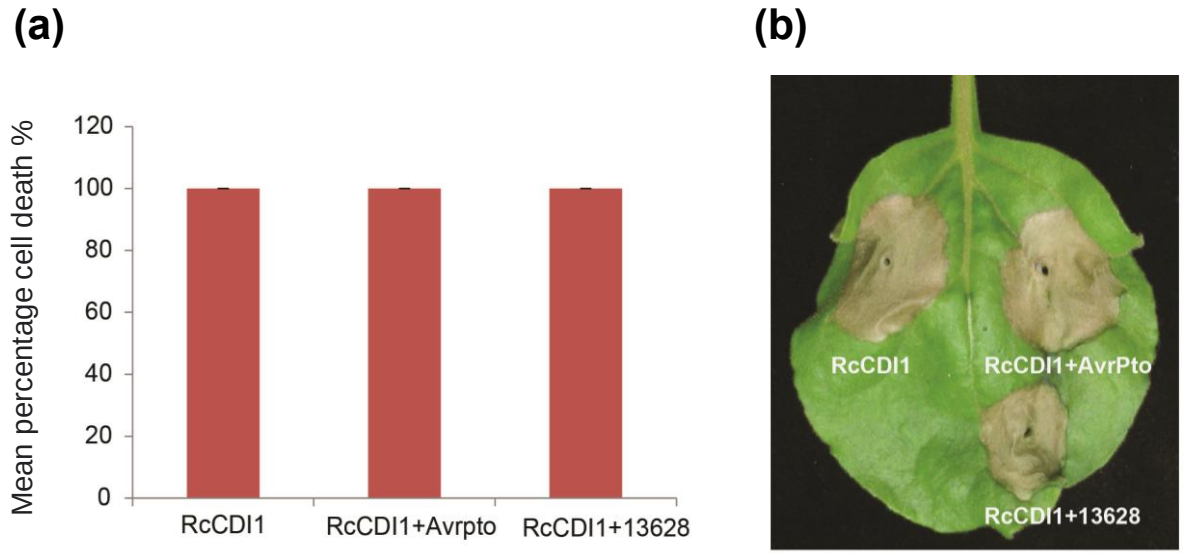

Figure 4.7 *Pseudomonas syringae* effector AvrPto and the *Phytophthora infestans* RxLR effector PIGT_13628 do not suppress RcCDI1-triggered cell death in *Nicotiana benthamiana*. (a) Percentage of HR at 7 days post co-infiltration using constructs encoding the indicated proteins. Co-expression of RcCDI1 with either AvrPto or PIGT_13628 did not suppress RcCDI1 induced cell death. Error bars indicate ± SD. (b) Representative leave taken 7 days post co-infiltration. Experiment was done with no less than seven plants with three leaves infiltrated per plant.

4.3 Discussion

RcCDI1 was considered previously to be a PAMP due to a number of observations including its cell death inducing activity in *N. benthamiana*; the fact that it is highly conserved in a wide range of different Ascomycete fungi; and the observation that RcCDI1homologues from *N. crassa*, *Z. tritici*, *M. oryzae*, *B. cinerea* and *S. sclerotiorum*, all induce cell death in Solanaceae. However, the mechanisms by which RcCDI1 is able to induce cell death remain unknown. Thus, in this study we aimed to gain further insight into the mechanisms involved in cell death response to RcCDI1. Trying to characterize RcCDI1-triggered cell death, we initially demonstrated that it was BAK1, SOBIR1 and SGT1 dependent.

We tested for BAK1 dependency because cell death induction in *N. benthamiana* required RcCDI1 to be present in the apoplast, suggesting that RcCDI1 detection may be mediated by a cell surface PRR, and BAK1 is a protein known to form regulatory complexes with multiple immune receptors (Greeff *et al.*, 2012). The observation that RcCDI1 cell death induction is BAK1 dependent suggests that a PRR-BAK1 complex is involved in RcCDI1 recognition.

RcCDI1 and PiINF1 triggered transcriptional upregulation of several PTI-related genes in *N. benthamiana* including *NbPTI5, NbACRE31,* and transcription factors *NbWRKY7* and *NbWRKY8*. However, silencing of *NbBAK1* prevented this upregulation, further confirming NbBAK1 involvement in RcCDI1 recognition, in the same way it has been stated in previous studies for PiINF1 (Chaparro-Garcia *et al.*, 2011). *NbWRKY7, NbWRKY8* and *NbACRE31* have also been shown to be induced by the flg22 peptide (Heese *et al.*, 2007; Hao *et al.*, 2014; Adachi *et al.*, 2015). The upregulation of some of the marker genes in *N. benthamiana* leaves infiltrated with *P. pastoris* EV (V5) or V5 peptide (Figure 4.2a,b), and its subsequent reduction in *BAK1* silenced plants, suggests that this control might contain other PAMPs triggering plant defence responses dependent on BAK1. Similarly, it has been shown that the same maker genes were upregulated in response to *P. infestans* PAMP cocktail (culture filtrate) in *N. benthamiana* plants (McLellan *et al.*, 2013). The above mentioned *N. benthamiana* maker genes activation, especially 0.5 h following infiltration with *P. pastoris* EV (V5) or V5 peptide can also be due to the wounding stress caused by leaf infiltration (Jaskiewicz *et al.*, 2010).

In addition, RcCDI11 slightly induced the expression of *HvPR1* and *HvAOS* genes connected with SA and JA signalling, respectively. Considering that this was a pilot experiment with only one biological repetition the observed transcript levels are likely to be within normal variation in transcript abundance. This is supported by the difference in *HvAOS* transcript abundance between barley leaves infiltrated with water and *P. pastoris* CS containing V5. The experiment needs repeating to see whether RcCDI1 has any significant effect on *HvPR1* and *HvAOS* transcripts.

The requirement of NbSOBIR1 for RcCDI1 recognition suggests that the receptor detecting RcCDI1 in *N. benthamiana* is likely to be an LRR-RLP (Liebrand *et al.*, 2014). The interaction of SOBIR1with RLPs was identified in previous studies (Liebrand *et al.*, 2013). It

was initially shown that the RLPs Cf-4 and Ve1 recognised the avirulence genes Avr4 and Ave1 from the fungal pathogens *C. fulvum* and *Verticillium dahliae*, respectively (Joosten *et al.*, 1994; De Jonge *et al.*, 2012). Interestingly, it was shown that the tomato homologues of the RLK AtSOBIR1 named SlSOBIR1 and SlSOBIR1-like interact with both RLPs, Cf-4 and Ve1, and are required for Cf-4 and Ve1-mediated resistance (Liebrand *et al.*, 2013). In addition, other studies showed that RLK FLS2-mediated resistance in AtSOBIR1 mutants was not compromised (Zhang *et al.*, 2013), supporting the hypothesis that SOBIR1 is involved in the defence responses mediated by RLPs. Taken together, the BAK1- and SOBIR1-dependent recognition of RcCDI1, along with its wide conservation across Ascomycetes, confirms that this fungal protein is a PAMP.

Another critical signalling component, NbSGT1, was also shown to be important for RcCDI1-triggered cell death. Similarly NbSGT1 was shown to be essential for the HR induced by the *P. infestans* PAMP PiINF1 as well as HR triggered by the co-expression of R proteins Rx, Pto, Cf-4, and Cf-9 with their corresponding pathogen effector in ETI responses, as all these responses were suppressed in *SGT1* silenced plants (Peart *et al.*, 2002).

RcCDI1 induced cell death in *N. benthamiana* in the presence of *P. infestans* RxLR effectors $Avr3a^{KI}$ and PexRD2, known to interact with CMPG1 and MAPKKKε proteins, respectively, to suppress plant innate immunity. This provides indirect evidence that RcCDI1-triggered cell death is independent of NbCMPG1 and NbMAPKKKε While the central regulator of PAMP-triggered immunity, NbBAK1, is common to responses to RcCDI1 and other PAMPs like oomycete elicitor PiINF1, NbCMPG1 appears not to be required for RcCDI1-triggered cell death, suggesting a different signalling pathway to that initiated by PiINF1.

In addition, RcCDI1 induced cell death in *N. benthamiana* was not affected by the presence of the *P. syringae* pv. tomato avirulence gene AvrPto or *P. infestans* RXLR effector PIGT_13628 previously shown to suppress immune responses triggered by Flg22 in tomato

and *A. thaliana* protoplasts (Zheng *et al.*, 2014). Our results suggest that while AvrPto can bind the Arabidopsis RLKs FLS2 and EFR and tomato LeFLS2 (Xiang *et al.*, 2008), it does not block RcCDI1 receptor in *N. benthamiana.* Shan *et al.*, (2008) proposed that instead of FLS2, AvrPto binds BAK1, thus interfering with ligand-dependent association of FLS2 with BAK1 during infection. They suggested that the association of AvrPto with FLS2 or EFR is weaker than with BAK1. In this chapter, it was shown that NbBAK1 was required for RcCDI1-triggered cell death using virus-induced gene silencing, so the fact that AvrPto did not suppress RcCDI1-induced cell death in *N. benthamiana* is in line with AvrPto binding the Arabidopsis FLS2 (Xiang *et al.*, 2008) and not BAK1, or was not effective enough in blocking *N. benthamiana* BAK1 to suppress RcCDI1-trigered cell death processes. In agreement with that AvrPto was shown to suppress the flg22- but not the PiINF1triggered cell death or the HR triggered by the co-expression of Avr9 and Cf9 in *N. benthamiana*, which are also BAK1 dependent (Kang *et al.*, 2004). The absence of suppression of RcCDI1 cell death mediated by PIGT_13628 suggests that while PIGT_13628 targets the flg22/FLS2 MAP kinase cascade, it does not suppress MAP kinase cascade leading to RcCDI1-mediated PCD, like it does not suppress MAP kinase cascades leading to Cf4- or INF1-mediated PCD (Zheng *et al.*, 2014).

In summary, demonstration of the direct role played by NbBAK1, NbSOBIR1 and NbSGT1 in *N. benthamiana* response to RcCDI1, and the upregulation of PTI marker genes in *N. benthamiana* upon RcCDI1 elicitation, extended our knowledge of the plant elements involved in the defence responses elicited by RcCDI1. The outcomes of this research are turning our attention towards some specific directions in the continuous search of resistance against ascomycetes in cereal crops.

Chapter 5. Combination of N-and C- terminal domains of RcCDI1 triggers cell death in *N. benthamiana*

5.1 Introduction

Plants have evolved specific defence mechanisms against microbial pathogens. In most cases, disease spread is limited upon the perception of MAMPs or PAMPs (Boller, 1995) by PRRs located on the cell surface, leading to PTI (Boller & Felix, 2009; Dodds & Rathjen, 2010). PAMPs are evolutionarily conserved across classes of microbes and are important to the microbial fitness. The best studied PAMPs are the bacterial flagellin, a protein subunit building up the filaments of the bacterial flagellum (Felix *et al.*, 1999), chitin, a fungal cell wall component (Kohler *et al.*, 2016), PGNs, a Gram-positive bacterial cell wall component (Gust *et al.*, 2007), and LPS, a Gram-negative bacterial cell wall component (Zeidler *et al.*, 2004).

The barley scald pathogen *R. commune* is considered a devastating pathogen not only for being highly destructive, but also because of its high diversity and its ability to change rapidly; greatly limiting the effectiveness of commonly used disease control measures (Newton *et al.*, 2001). Alternative disease control strategies have been implemented with some level of success (McDonald, 2015), but an increased understanding of the virulence factors and the molecular mechanisms utilised by *R. commune* to cause disease is required to effectively combat this pathogen. For that matter, in our work, a new proteinaceous PAMP termed RcCDI1 was identified in Ascomycete fungi (Chapter 3-4). RCDI1 homologues from *R. commune, B. cinerea, S. sclerotiorum, Z. tritici, M. oryzae* and *N. crassa* were shown to trigger cell death in *N. benthamiana* and other solanaceous plants. Here, we searched for the protein domain (s) required for the cell death triggered by RcCDI1 in *N. benthamiana* and we aimed to elucidate the importance of a relatively conserved 16 amino acid domain within the N-terminal domain of CDI1 proteins.

It is widely known that proteins have structural and functional domains as their basic units (Feldman, 2012). Proteins can be formed by single or several domains that play important roles in the protein function (Aroul-Selvam *et al.*, 2004). The first description of protein domains was done by Donald Wetlaufer in 1973 (Wetlaufer, 1973) and subsequently many different strategies have been employed to annotate a huge range of protein domains (Feldman, 2012). Several microbial protein domains have been described as being perceived by the plant immune system in a process believed to be crucial for the activation of effective plant defence responses. For instance, PAMPs possess highly conserved domains specifically recognised by the plants, as it is the case for flg22, a conserved 22 amino acid motif within the N-terminal region of bacterial flagellin protein, which is recognised by FLS2 in Arabidopsis (Felix *et al.*, 1999), while another flagellin derived peptide flgII-28 is recognised by the flagellin-sensing 3 receptor (FLS3) in certain solanaceous plants (Hind *et al.*, 2016). Another example is the N-acetylated 18 amino acid motif from the EF-Tu, bacterial protein recognised in Arabidopsis and plants from Brassicaceae family by the plant receptor EFR (Kunze *et al.*, 2004). In the case of non-proteinacious PAMPs, it was shown that the cell surface receptor CEBiP recognises chitin oligomers, especially heptamer-octamer, to activate chitin-triggered immunity (Kaku *et al.*, 2006; Hayafune *et al.*, 2014). In the same way, chitosan, a deacetylated variant of chitin, and especially its pentamers and heptamers, have been shown to induce plant defence responses (Akiyama *et al.*, 1995).

It has also been shown that domain deletion analysis is a good method for the understanding of protein functional domains in protein-protein interactions. Several studies have been performed for several proteins from plant and pathogens. In the case of RxLR effectors, for example, the C-terminus of *Plasmopara viticola* effector PvRxLR28 was shown to be required for programmed cell death (PCD) suppression in *N. benthamiana* (Xiang *et al.*, 2016); the N-terminal motif of *P. sojae* effector Avh241 was shown to be required for cell-death inducing activity (Yu *et al.*, 2012); 75 amino acids from the C-terminal domain of *P.*

infestans effector AVR3aKI was shown to be required for Avr3a/R3a mediated cell death (Bos *et al.,* 2006). The same studies have been carried out for Crinkler effectors (CRN). For example, a234 amino acid region in C-terminal domain from CRN2 secreted protein was shown to be responsible for the cell death induction in *N. benthamiana* (Haas *et al.*, 2009); the 133-411 aa fragment from *P. sojae* effector PsCRN63 is required for cell death induction, whereas the 132-424 aa fragment of PsCRN115 effector is sufficient for suppression of the cell death triggered by PsCRN63 in *N. benthamiana* (Liu *et al.*, 2011). All these examples demonstrating the role of important protein domains during plant-microbe interactions turned our interest towards the elucidation of the RcCDI1 domains involved in recognition. Therefore, RcCDI1 was divided into different domains, and they were individually expressed in *N. benthamiana*. N-and C-terminal domains were found to be important for the induction of cell death. Moreover, the co-expression of N- and C-terminal domains of RcCDI1 increased the percentage of *N. benthamiana* infiltration sites responding with a cell death phenotype. We also tested constructs expressing truncated versions of RcCDI1 to establish the smallest part of RcCDI1 required for induction of cell death in *N. benthamiana.* Furthermore, cell death was not shown for the deletion variant of RcCDI1 lacking a 16 amino acid motif at the most conserved region amongst CDI1 homologues.

In addition to this, we were interested to evaluate the CDI1 homologue of *B. graminis* f.sp. *hordei* and its potential recognition in *N. benthamiana*. The Ascomycete *B.graminis* f.sp. *hordei* is an obligate biotrophic pathogen and the causal agent of powdery mildew in barley (Koeck *et al.*, 2011). We observed that *B. graminis* f.sp. *hordei* homologue of CDI1 did not induce cell death in *N. benthamiana* when it was over-expressed either with or without signal peptide. Therefore, we were interested to elucidate the protein sequence divergence that prevents this *B. graminis* homologue from causing cell death in *N. benthamiana*.

In summary, the overexpression of different domains of RcCDI1 provided significant insights into the part of the protein required for the induction of cell death in *N. benthamiana*.

Moreover, the fact that CDI1 from *B. graminis* homologue did not induce cell death and the work described in this chapter and in previous chapters on characterizing CDI1 from *R. commune* and its homologues could provide important information about the mechanisms used by *R. commune* to lead a successful infection.

5.2 Results

5.2.1 Expression of RcCDI1 domains in *N. benthamiana*

Protein sequence alignments of CDI1 homologues from *R. commune*, *B. cinerea*, *S. sclerotium*, *Z. tritici*, *M. oryzae* and *N. crassa*, that were shown to trigger cell death in *N. benthamiana* suggested that RcCDI1 can be split into three domains: N-terminal, internal and C-terminal (Figure 5.1). The most conserved part of the N-terminal domain is an ~35 aa peptide, from 30 to 65 aa (Figure 5.1). The internal domain is the least conserved part of the protein. To identify the part of RcCDI1 that induces cell death in *N. benthamiana*, truncated versions as well as different parts of RcCDI1 were transiently expressed in *N. benthamiana*. Six constructs were generated, RcCDI1$_{1\text{-}99}$, RcCDI1$_{1\text{-}88}$, RcCDI1$_{1\text{-}59}$, RcCDI1$_{44\text{-}59}$, RcCDI1$_{89\text{-}155}$, RcCDI1$_{156\text{-}200}$ fused to C-terminal mRFP from a conventional binary vector pK7RWG2. They were expressed with the RcCDI1 native signal peptide to ensure their secretion into the apoplast to test for their ability to trigger cell death in *N. benthamiana*. Individual expression of constructs RcCDI1$_{1\text{-}99}$, RcCDI1$_{1\text{-}88}$, RcCDI1$_{1\text{-}59}$, RcCDI1$_{44\text{-}59}$, RcCDI1$_{89\text{-}155}$ and RcCDI1$_{156\text{-}200}$ induced cell death in 48, 8, 23, 13, 4 and 42 % of *N. benthamiana* infiltration sites respectively (Figure 5.2a). Expression of pK7RWG2 empty vector did not induce cell death and full length RcCDI1 triggered cell death in 85% of the infiltration sites (Figure 5.2a).

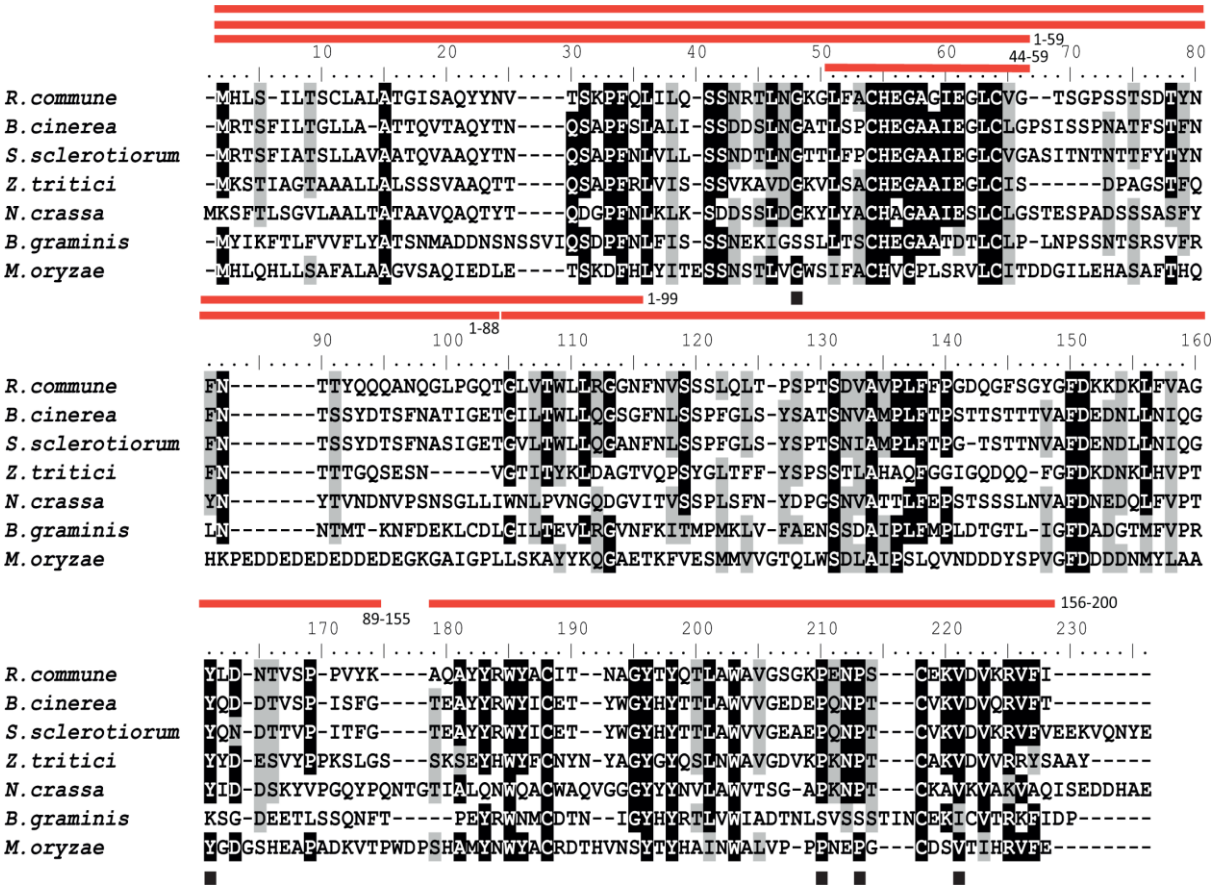

Figure 5.1 ClustalW alignment of protein sequences of *R. commune* CDI1 with its homologues from other Ascomycete fungi. Red lines mark the division of RcCDI1 into domains ($RcCDI1_{1\text{-}99}$, $RcCDI1_{1\text{-}88}$, $RcCDI1_{1\text{-}59}$, $RcCDI1_{44\text{-}59}$, $RcCDI1_{89\text{-}155}$, $RcCDI1_{156\text{-}200}$). Black squares indicate the amino acids conserved in six CDI1 homologues from different fungal species shown to trigger cell death in *N. benthamiana*, and not in *B. graminis*, suggesting that they might be crucial for CDI1 recognition in *N. benthamiana*.

5.2.2 Co-expression of N- and C-terminal domains of RcCD1 increased the percentage of *N. benthamiana* infiltration sites responding with cell death

To further investigate which part of the RcCDI1 is required for triggering cell death in *N. benthamiana* the fragments RcCDI1$_{1-99}$ and RcCDI1$_{156-200}$,that were shown to induce cell death in the highest proportion of infiltrated leaves (Figure 5.2b), were co-infiltrated. The co-expression of these two parts of RcCDI1 resulted in a much higher percentage of cell death (71%) compared to the cell death triggered when these two constructs were expressed individually, RcINS1$_{1-99}$ (48%) and RcINS1$_{156-200}$ (42%) (Figure 5.2b) and made it comparable to the 85% cell death in case of the full length RcCDI1 (Figure 5.2b). At the same time co-agroinfiltration of RcCDI1$_{1-88}$ construct with RcCDI1$_{156-200}$ resulted in only 38% of cell death, similar to the proportion of infiltration sites that responded to RcCDI1$_{156-200}$ (Figure 5.2b).

Immunoblots of all protein samples taken from *N. benthamiana* leaves indicated that lower percentage of *N. benthamiana* infiltration sites responding with cell death to some of the constructs was not due to a much lower amount of protein expressed, as most of them were expressed at similar levels. Protein expression was very high for the full length RcCDI1 and very low for RcCDI1$_{156-200}$ so they could not be included in the western blot shown (Figure 5.2c). Protein sequences of RcCDI1$_{156-200}$, RcCDI1$_{89-155}$, RcCDI1$_{1-59}$ and RcCDI1$_{1-88}$ suggest that the molecular weights of the mRFP-tagged mature proteins should be 32, 35, 32 and 35 kDa, respectively, whereas immunoblot revealed higher molecular weight for each of these RcCDI1 domains. As previously mentioned in chapter 3, the molecular weights of RcCDI1 and its homologs were also higher in the western blot. This was attributed to post-translational modification of glycosylation by the use of *P. pastoris* expression system. It can also be the case of the higher molecular weights shown by the RcCDI1 domains in the western (see below), with glycosylation occurring *in planta*.

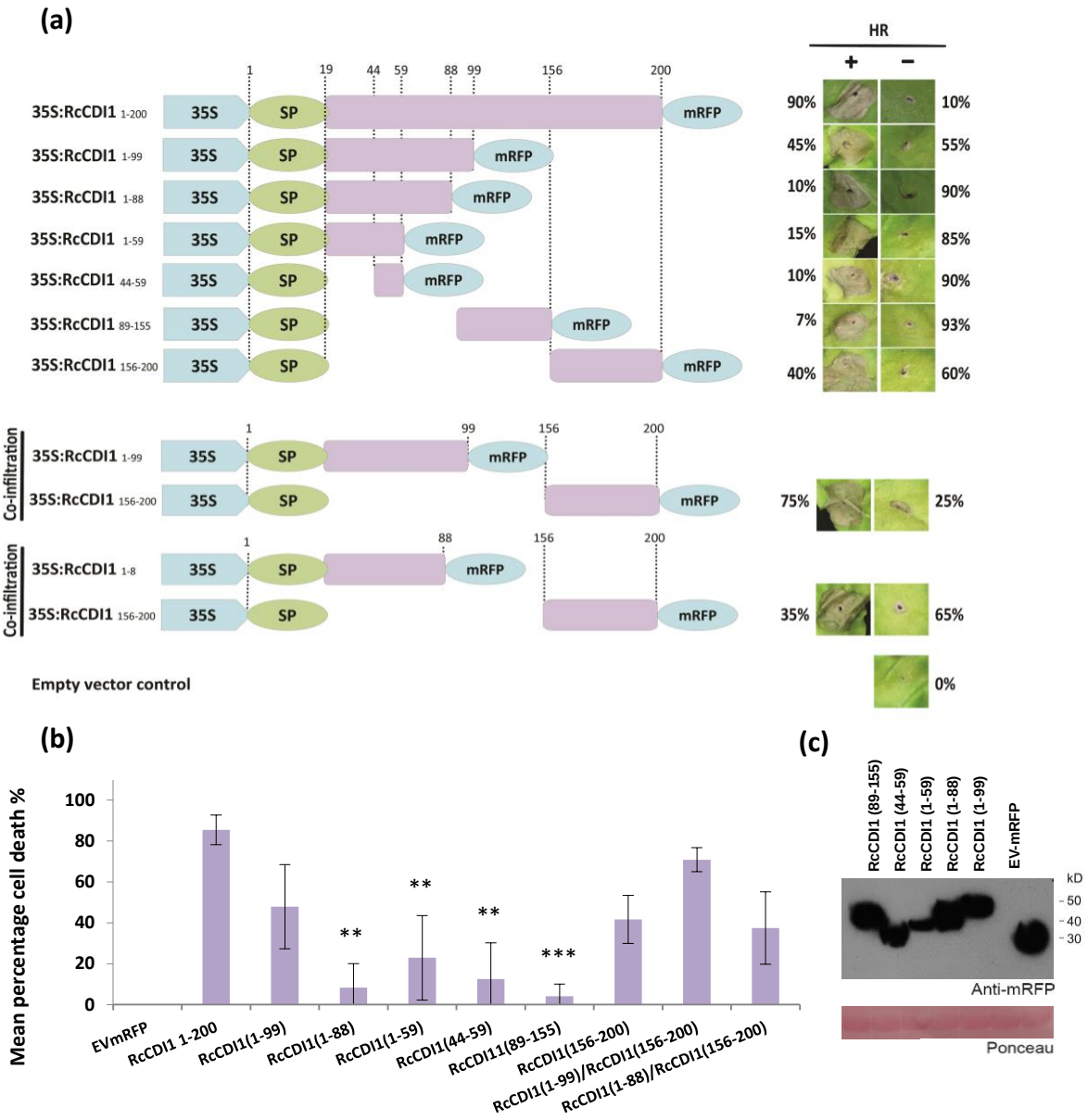

Figure 5.2 Expression and co-expression of RcCDI1 domains induces cell death in *N. benthamiana*. (a) RcCDI1 was divided into domains and each of them was expressed with a C-terminal mRFP fusion from a pK7RWG2 vector. The expression of constructs RcCDI1$_{1\text{-}99}$, RcCDI1$_{1\text{-}88}$, RcCDI1$_{1\text{-}59}$, RcCDI1$_{44\text{-}59}$, RcCDI1$_{89\text{-}155}$ and RcCDI1$_{156\text{-}200}$ induced cell death in 48, 8, 23, 13, 4 and 42% of *N. benthamiana* infiltration sites respectively. The co-expression of RcCDI1$_{1\text{-}99}$ and RcCDI1$_{156\text{-}200}$ induced cell death in 71% of the cases. The co-expression of RcCDI1$_{1\text{-}88}$ construct with RcCDI1$_{156\text{-}200}$ induced cell death in 38% of the infiltrated leaves. The expression of pK7RWG2 empty vector did not induce cell death and full length RcCDI1 triggered cell death in 85% of the infiltration sites. (b) The percentage of infiltration sites developing cell death in *N. benthamiana* leaves at seven dpi with constructs encoding the indicated domains of RcCDI1. The experiment was performed three times, each time with at least eight plants for each construct. Error bars indicate ± SD. (c) Immunoblot of proteins from *N. benthamiana* leaves transiently expressing, RcCDI1$_{89\text{-}155}$, RcCDI1$_{44\text{-}59}$, RcCDI1$_{1\text{-}59}$, RcCDI1$_{1\text{-}88}$ and RcCDI1$_{1\text{-}99}$ with C-terminal mRFP fusion from a pK7RWG2 vector. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Holm-Sidak test; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

5.2.3 RcCDI1 with 16 amino acid deletion does not trigger cell death in *N. benthamiana*

The N-terminal domain of RcCDI1 protein sequence contains a highly conserved 16-amino acid stretch (Figure 5.3a). To further investigate the involvement of this part of RcCDI1 in triggering cell death in *N. benthamiana* an RcCDI1 construct missing the 16 amino acids motif fused to a C-terminal mRFP was generated. Hereafter, the deletion construct is called RcCDI1Δ^{16}. The deletion mutant was transiently overexpressed in *N. benthamiana* leaves using *A. tumefaciens* alongside the full length RcCDI1 as a positive control. As expected, the full length RcCDI1 protein conferred cell death in 80% of the infiltration sites, with symptoms appearing at 3 dpi. In contrast, *N. benthamiana* leaves expressing RcCDI1Δ^{16} did not show cell death (Figure 5.3b,c). In addition, the empty vector PK7RWG2 did not induce cell death. These results suggest that this conserved 16 amino acids motif or some of the amino acids within it might be required for the induction of plant defence response in *N. benthamiana*. The lack of cell death for RcCDI1Δ^{16} is not due to low levels of expression because high protein levels were detected by Western blot. It is important to note that the size of the protein RcCDI1Δ^{16} is smaller than the expected size ~ 46 kDa (Figure 5.3d).

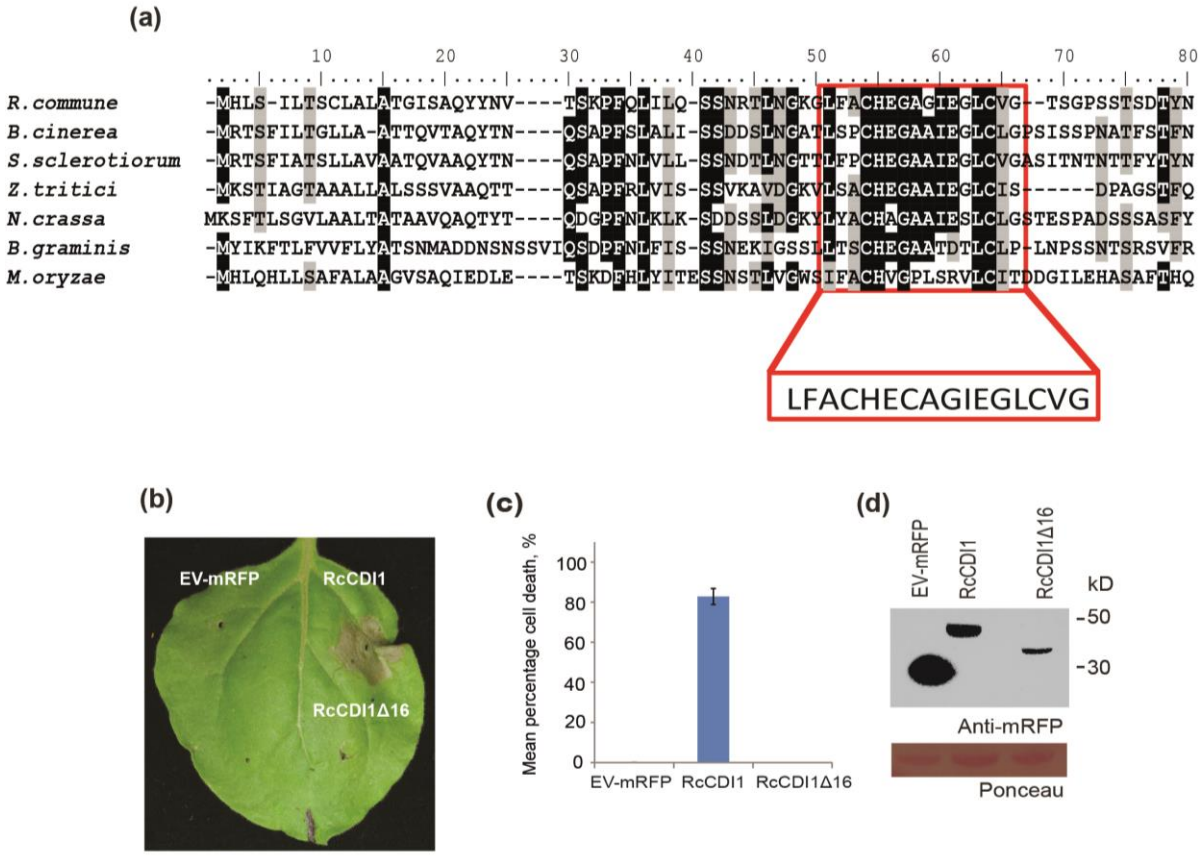

Figure 5.3 Expression of RcCDI1 missing an 16-amino acid motif (RcCDI1Δ^{16}) does not induce cell death in *N. benthamiana*. (a) 16 amino acid motif showed in the alignment of the protein sequence of *R. commune* CDI1 with its homologues from other fungal species (b) Representative *N. benthamiana* leaf six dpi using pK7RWG2 constructs expressing the full length RcCDI1 with and without a conserved 16 amino acids motif (RcCDI1Δ^{16}) with C-terminal mRFP fusion. (c) The percentage of infiltration sites developing a clear cell death in *N. benthamiana* leaves at six dpi mediated by a pK7RWG2 vector control (EV) expressing mRFP or RcCDI1 with C-terminal mRFP fusion with or without 16 amino acid motif within the N-terminal domain. (d) Immunoblot of proteins from *N. benthamiana* leaves transiently expressing the indicated proteins with C-terminal mRFP fusion from a pK7RWG2 vector. The experiment was performed two times, each time with at least five plants for each construct. Error bars indicate ± SD.

5.2.4 *B. graminis* homologue of RcCDI1 does not induce cell death in *N. benthamiana*

It was previously shown in chapter 3 that RcCDI1 from *R. commune* and its homologues from hemibiotrophic (*Z. tritici* and *M. oryzae)* and necrotrophic (*B. cinerea and S. sclerotiorum)* fungal plant pathogens, and a saprophyte (*N. crassa)* were able to induce cell death in *N. benthamiana*. To investigate whether the RcCDI1homologue from a biotrophic barley pathogen *B. graminis* (BgCDI1) can also induce cell death in *N. benthamiana*, full length and

truncated, lacking a signal peptide, versions of BgCDI1 were transiently expressed as a C-terminal mRFP fusion from the vector pK7RWG2. All constructs generated products of expected size (Figure 5.4c). While, as expected, the RcCDI1 from *R. commune* expressed with the endogenous signal peptide used as a positive control triggered strong cell death (94%) (Figure 5.4a,b), neither BgCDI1 expressed with (SP-BgCDI1) or without (BgCDI1) the endogenous signal peptide triggered cell death in any of the infiltration sites (Figure 5.4a,b). These data indicated that BgCDI1 from *B. graminis* is not recognised in *N. benthamiana* leaves when the protein is expressed either inside the *N. benthamiana* cells or secreted into the apoplast.

Comparison of BgCDI1 protein sequence to its homologues from *R. commune, B. cinerea, S. sclerotium, Z. tritici, M. oryzae* and *N. crassa,* that triggered cell death in *N. benthamiana,* highlighted 5 conserved amino acids, indicated by black squares in Figure 5.1, that are different in BgCDI1. One of these amino acids is located in the N-terminal domain, another one towards the end of the middle part of the protein and the last three, located in the last conserved part of the C-terminal domain. As the internal part of the CDI1 was shown not to be essential for triggering cell death in *N. benthamiana,* it's the remaining 5 amino acids that are likely to be crucial for CDI1 recognition in *N. benthamiana.*

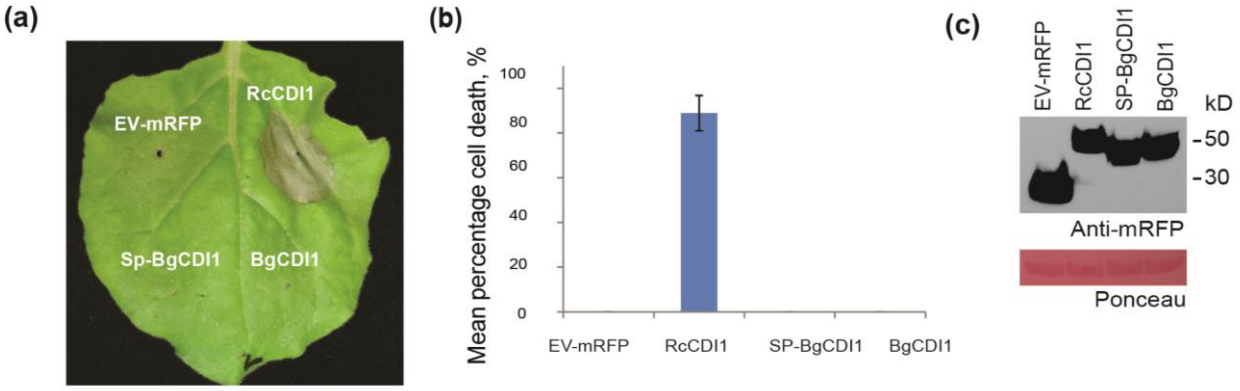

Figure 5.4 Expression of full length CDI1 from *B. graminis* (BgCDI1) does not induce cell death in *N. benthamiana*. (a) Representative *N. benthamiana* leaf six dpi using pK7RWG2 construct expressing the full length BgCDI1 with and without signal peptide with C-terminal mRFP fusion. (b) The percentage of infiltration sites developing a clear cell death in *N. benthamiana* leaves at six dpi mediated by a pK7RWG2 vector control expressing mRFP (EV-mRFP), full length RcCDI1 or BgCDI1 with C-terminal mRFP fusion with or without signal peptide. (c) Immunoblot of proteins from *N. benthamiana* leaves transiently expressing the indicated proteins with C-terminal mRFP fusion from a pK7RWG2 vector. The experiment was performed two times, each time with at least six plants for each construct. Error bars indicate ± SD.

5.3 Discussion

Many different molecules from both the pathogen and the plant are released during their interaction defining disease outcomes. Proteinaceous and non-proteinaceous PAMPs have been characterised over the years as conserved molecules with essential functions for microbe persistence and survival (Medzhitov & Janeway, 1997; Nürnberger & Brunner, 2002; Boller & Felix, 2009; Zhang & Zhou, 2010; Zipfel & Robatzek, 2010). Cell wall β-glucans, chitin, oligosaccharides, glycoproteins, flagellin-flg22 and EF-tu-elf18 are the best studied PAMPs (Felix *et al.*, 1999; Zeidler *et al.*, 2004; Kunze *et al.*, 2004; Gust *et al.*, 2007; Kohler *et al.*, 2016; Fesel & Zuccaro, 2016).

RNA sequencing of barley plants infected with *R. commune* allowed the identification of RcCDI1, a novel fungal PAMP (Chapter 3). RcCDI1 was shown to be upregulated at an early infection stage (3 dpi) of barley and was shown to be able to trigger cell death in solanaceous plants but not in monocots. Besides, RcCDI1homologues from other fungal species from the ascomycete group were also shown to trigger cell death in *N. benthamiana*. Nevertheless,

little is known about the RcCDI1 epitopes involved in the activation of cell death in *N. benthamiana.*

To identify the RcCDI1 protein domain(s) recognised in *N. benthamiana*, RcCDI1 was divided into 3 domains, N-terminal, internal and C-terminal, with the generation of three constructs ($RcCDI1_{1-99}$, $RcCDI1_{89-155}$, $RcCDI1_{156-200}$). The cell death caused by these fragments of RcCDI1 was evaluated by transient overexpression in *N. benthamiana*. Individual expression of the constructs induced cell death in *N. benthamiana* but at different levels. The N-terminal, $RcCDI1_{1-99,}$ and the C-terminal, $RcCDI1_{156-200,}$ domains induced the highest percentage of cell death, 48% and 42% respectively, compared to just 4 % of cell death induced by the internal domain, $RcCDI1_{89-155}$. However these levels were still only about half the level of cell death triggered by the expression of full length RcCDI1 (85%). Interestingly the co-expression of these two domains in *N. benthamiana* led to almost doubling of the cell death percentage observed (71%). This confirms the importance of the conserved N-and C-terminal domains of RcCDI1 in plant recognition.

Our results suggest that unlike flagellin and EF-Tu containing a single epitope required for full recognition by a cognate PRR, RcCDI1 contains a complex epitope or 2 epitopes, both of which have to bind to the receptor for full recognition to occur. The reasons for the high percentage of cell death observed during the co-expression of these two domains are still unknown but it suggests that the N-and C-terminal domains of RcCDI1 could be coming together during the folding of the intact RcCDI1 and close proximity of $RcCDI1_{1-99}$ and $RcCDI1_{156-200}$ within apoplast following their expression and secretion allowing simultaneous binding of the receptor to epitopes from both domains. Another possibility might be RcCDI1 dimerization *in planta*, although western blot analysis of proteins from *N. benthamiana* leaves transiently expressing the RcCDI1 did not provide support for RcCDI1dimerization. Dimerization *in planta* has been shown previously to be important for the biological functions of the protein effectors CRN63, CRN8 and AvrBS3 from the pathogens *P. sojae*, *P. infestans*

and *Xanthomonas campestris* pv. *vesicatoria*, respectively (Gürlebeck *et al.*, 2005; van Damme *et al.*, 2012; Li *et al.*, 2016). Another explanation for the high percentage of cell death after the co-expression of these N- and C-terminal domains, is focused in the action of two potential receptors involved in RcCDI1 recognition, fusing together in a complex. This has been previously described during the interaction between chitin oligomers and the receptor CEBiP, indicating that two CEBiP molecules simultaneously bind to the chitin oligomer showing a "sandwich-like" dimerization of CEBiP (Hayafune *et al.*, 2014).

The cell death observed when over-expressing truncations of the N-terminal domain of RcCDI1 to produce RcCDI1$_{1\text{-}88}$, missing a fairly conserved 9 amino acid peptide at the end of the N-terminal domain, suggests that this peptide, or some amino acids within it, is essential for RcCDI1 recognition as in its absence the percentage of infiltration sites developing cell death in *N. benthamiana* leaves dropped from 48 to 8 % (Figure 5.2). Both RcCDI1$_{44\text{-}59}$, the most conserved 16-amino acid region within the N-terminal domain and RcCDI1$_{1\text{-}59}$, a highly conserved part of the N-terminal domain containing RcCDI1$_{44\text{-}59,}$ induced cell death in a very small proportion of infiltration sites, suggesting that while these part of the RcCDI1 might be required for RcCDI1 recognition, it is not sufficient to trigger plant cell response comparable to that triggered by intact RcCDI1 or even RcCDI1$_{1\text{-}99}$.

In addition, further investigation was done to describe the importance for cell death inducing activity of the highly conserved 16-amino acid region within the N-terminal domain of RcCDI1. Overexpression of RcCDI1 missing 16 amino acids (RcCDI1Δ^{16}) in *N. benthamiana* leaves did not induce cell death, suggesting the importance of this peptide for the induction of defence responses in *N. benthamiana*. It has previously been shown that cysteines are involved in disulphide bond formation, playing an important role in protein stability and folding (Joosten, 1997; Thangudu *et al.*, 2008). The apoplast is a very hostile protease-rich environment (Catanzariti *et al.*, 2007) and PAMP recognition by PRRs occurs in this extracellular space (Doehlemann & Hemetsberger, 2013). Thus apoplastic proteins have

cysteine residues as a valuable resource to persist under the harsh apoplastic conditions (Joosten, 1997). *R. commune* RcCDI1 protein contains 4 cysteine residues with two of them located at the beginning and at the end of the 11-amino acid most conserved section within the 16-amino acid motif. The deletion of the entire motif containing these two cysteines may have substantially altered RcCDI1 structure or stability leading to a consequent loss of recognition by the plant. This hypothesis is supported by the smaller size observed for RcCDI1Δ^{16} in the western blot compared to the observed size of the full length RcCDI1.

Similarly, little or no necrosis inducing activity in tomato plants was shown by the apoplastic effector Avr9 from *C. fulvum* when its protein size was reduced and its cysteines were modified to avoid the formation of disulphide bridges, highlighting the cysteine importance for apoplastic protein activities (Van den Hooven *et al.*, 2001). In addition, cell death inducing activity by the apoplastic effector Avr4 in Cf4 containing tomato plants was lost due to substitution of the cysteine residues in a mutated Avr4 protein (Lida *et al.*, 2015).

It was previously shown that RcCDI1 homologues from the fungal pathogens *Z. tritici*, *M. oryzae, B. cinerea, S. sclerotiorum,* as well as a saprophyte *N. crassa* triggered cell death in *N. benthamiana* (chapter 3). Here, the over-expression of full length and truncated (lacking a signal peptide) versions of RcCDI1 from *B. graminis* (BgCDI1) expressed either with or without signal peptide, did not induce cell death in *N. benthamiana,* suggesting a divergence between the *B. graminis* sequence and the sequences from the other homologues that induce cell death. *B. graminis* is a biotrophic pathogen of barley, which requires living plant cells to survive and reproduce (Dickman & de Figueiredo, 2011). Cell death is a plant defence mechanism against this type of pathogens, stopping the fungus from growing and colonizing (Govrin & Levine, 2000). Although like *R. commune, Z. tritici, M. oryzae, B. graminis* does not infect *N. benthamiana* the absence of cell death in *N. benthamiana* from BgCDI1 could be at least to some extent associated with this pathogen's biotrophic lifestyle.

The inability of BgCDI1 to trigger cell death in *N. benthamiana* suggests that it is missing the crucial amino acids recognised by the putative RcCDI1 receptor in *N. benthamiana.* This could be explained by *B. graminis* evolutionary effort for the modification of BgCDI1 protein sequence to avoid plant detection, correlated with its requirement of live host tissue to survive (Orton & Brown, 2016). Similarly, some microbial species showed divergence in the flg22 motif (the most conserved motif of bacterial flagellin), as it is the case for *Agrobacterium* and *Rhizobium* flagellin homologues that did not induce defence responses in tomato and *A. thaliana* (Bauer *et al.*, 2001; Boller & Felix, 2009). Thus, even if PAMPs are broadly conserved between species they can also undergo evolutionary processes to avoid plant recognition and the consequent activation of PTI responses, and this can be the case for BgCDI1 protein sequence.

Comparing protein sequences of 6 CDI1 homologues triggering cell death with the sequence from *B. graminis,* we found that five amino acids might contribute to plant-mediated recognition processes (Figure 5.1). Future experiments involving single-residue substitutions will help to evaluate the role of these amino acids in plant recognition. In addition, the functionality of the predicted secretory signal peptide from the CDI1 protein from *B. graminis* must be checked, to discard the possibility that the absence of cell death was due to the absence of BgCDI1 in the plant apoplast. This might be achieved using confocal microscopy to confirm BgCDI1-mRFP secretion. Alternatively the BgCDI1 could be expressed with the SP from RcCDI1, previously shown in chapter 3 to be involved in the secretion of RcCDI1 to the extracellular space in *N. benthamiana* where it was recognised.

In summary, the division of RcCDI1 into different domains and their expression in *N. benthamiana,* led to the identification of the N- and C-terminal domains of RcCDI1 as the important domains required for the induction of cell death. In addition CDI1 protein from *B. graminis* did not induce cell death in *N. benthamiana*. This allowed us to narrow down the focus of our future research into the identification of those specific amino acids involved in

the recognition. Further studies on these domains and the validation of the specific amino acids of *B. graminis* that lead to the absence of cell death, constitute a valuable tool to provide new insights into the specific ways by which RcCDI1 is being recognised. Further studies on this novel PAMP involve the identification of the plant receptor involved in RcCDI1 recognition, and with the use of biotechnological tools, engineering non-host resistance to this devastating crop pathogen.

Chapter 6. Characterization of *Rc2* and *Rsu3_07158* candidate pathogenicity factors from *R. commune.*

6.1 Introduction

Microbial genome and transcriptome sequencing is at present one of the most important approaches used in molecular biology (Imam *et al.*, 2016), providing insights into different molecular processes and biochemical activities within an organism. Whole genome sequences for a broad range of plant pathogens have now become available, elucidating the presence of predicted secreted protein families with suggested roles in pathogenicity (Kim *et al.*, 2016). For example, the genome sequence from the bacterium *Xylella fastidiosa* led to the identification of pectolytic enzymes involved in the effective colonization by the pathogen or RTX-like proteins which are toxins that act as virulence factors (Simpson *et al.*, 2000). It is also the case for *P. infestans* with the identification of cytoplasmic effectors, corresponding to RXLR and Crinkler (CRN) families (Haas *et al.*, 2009). For *S. sclerotiorum* and *B. cinerea* pathogens, enzymes involved in the generation of reactive oxygen species (ROS), NADPH oxidase encoding genes and carbohydrate-active enzymes (CAZymes) required for plant cell wall degradation were found by genomic sequencing efforts (Amselem *et al.*, 2011). In addition, transcriptome sequencing involves the analysis of all the RNA transcripts, including mRNAs, and in some cases non-coding RNAs and small interfering RNAs (Wang *et al.*, 2009). Transcriptome sequencing is a common strategy, essential for the identification of functional genomic elements; helping to describe gene regulation networks under different conditions, as, for example, during plant pathogen interactions resulting in disease (Wang *et al.*, 2009). Moreover, the structure of the genes including their translation initiation factors, alternative splicing pattern, among others can be identified using transcriptomic analyses (Wang *et al.*, 2009).

R. commune is one of the most destructive pathogens of barley (Avrova & Knogge, 2012), but our knowledge remains limited when considering *R. commune* pathogenicity factors. Considering *R. commune* secreted proteins, only the action of a small family of necrosis inducing peptides (NIPs) has yet been elucidated (Wevelsiep *et al.*, 1991). Therefore, a gain in knowledge of the *R. commune* infection biology and the molecules involved in *R. commune* – plant interactions are crucial for the development of the more effective disease management strategies.

In order to acquire a deep understanding of the proteins from *R. commune* active during infection, sequencing of mRNA from epidermal strips of barley leaves infected with *R. commune* was performed (Penselin *et al.*, 2016), allowing the identification of two transcripts called *R. commune* 2 (Rc2) and Rsu3_07158 coding for two small secreted proteins of 74 and 174 amino acids respectively. These transcripts were highly abundant during barley infection leading us to hypothesize that both proteins could constitute novel *R. commune* effectors.

Barley genotypes showing high level of resistance to *R. commune* over 3 years of field trials were screened for recognition of individual effectors using the BSMV-based expression system (Kanyuka *et al.*, unpublished). Overexpression of the *Rc2* allele from *R. commune* strain L2A in the barley landrace SLB-10-009, considered one of the most resistant lines evaluated, induced necrotic lesions, indicating a potential recognition of the candidate effector Rc2. Virulence tests were carried out in the barley line SLB-10-009 and susceptible cv Optic to determine the correlation between the virulence shown by the sequenced *R. commune* strains with their nucleotide sequence changes in this potential avirulence gene, Rc2.

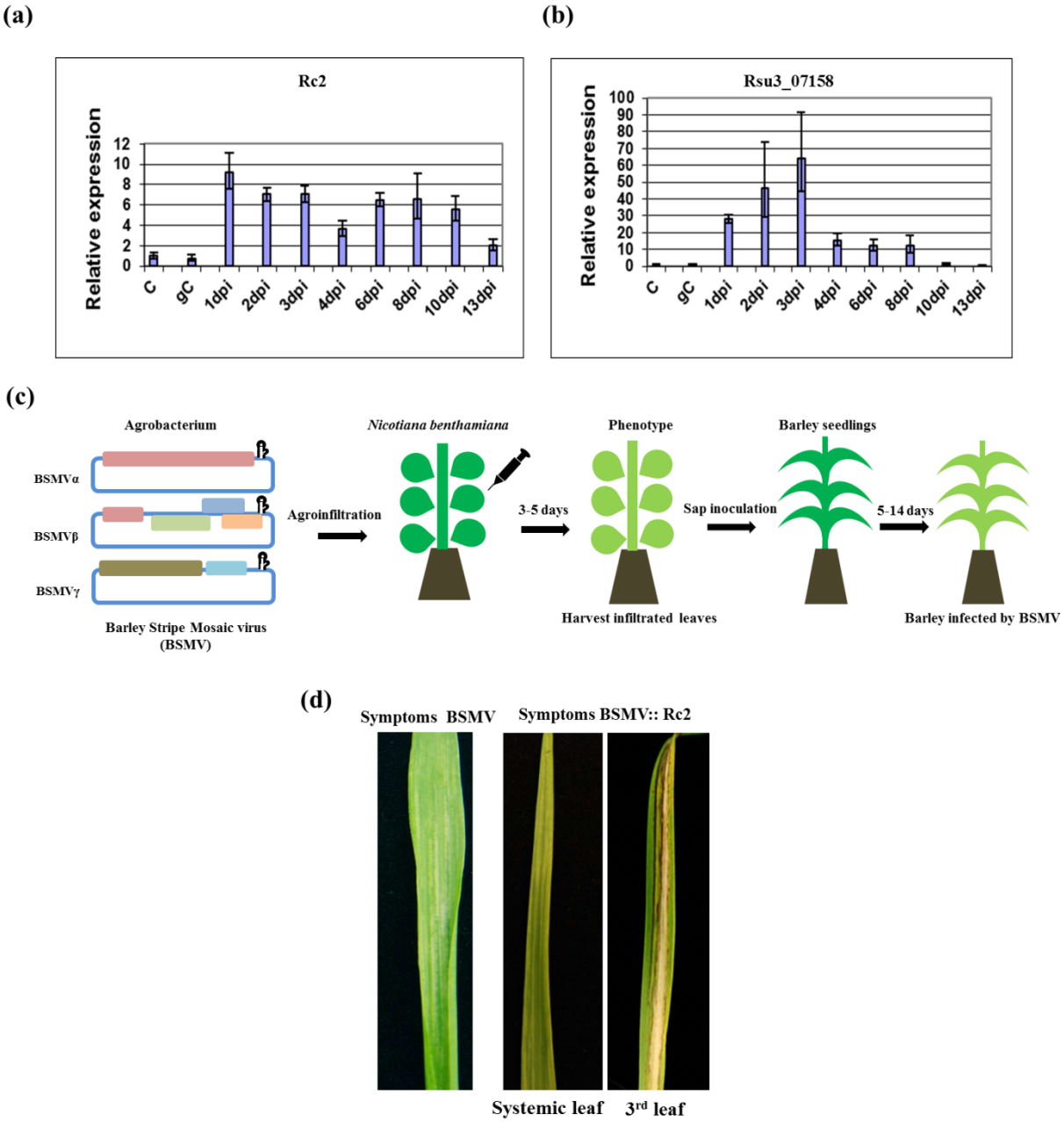

Figure 6.1 *Rhynchosporium commune* genes *Rc2* and *Rsu3_07158* are upregulated during infection in barley leaves, and Rc2 triggered the cell death phenotype in the line SBL 10-009 when expressed using Barley stripe mosaic virus (BSMV)-expression system. (a,b) *Rc2* and *Rsu3_07158* transcripts abundance in *R. commune* germinated conidia and at 1, 2, 3, 4, 6, 8, 10 and 13 days post-inoculation (dpi) of susceptible barley cv Optic with *R. commune* relative to its level in conidia which was assigned the value of 1.0. Error bars represent 95% confidence intervals calculated using three technical replicates for each sample within the RT-PCR assay. (c) BSMV technique. *N. benthamiana* leaves agroinfiltrated with BSMV:Rc effector are used as a source of inoculum to infect barley seedlings. (d) Cell death phenotype on systemic and 3rd leaves shown by *R. commune* effector Rc2 expressed in the barley line SLB-10-009 using the BSMV system (leaves on the right). The typical BSMV symptoms in all the barley genotypes tested include mild viral mosaic phenotype (barley leaf on the left). Photos were taken at 14 dpi. This work was carried out by Dr. Anna Avrova and her group at The James Huton Institute and Dr. Kostya Kanyuka and his group at Rothamsted Research, prior to the beginning of my PhD but I composed the picture.

In addition, we were interested to characterize the gene *Rsu3_07158* which was predicted to contain an InterPro motif IPR021054, coding for a Cell wall mannoprotein 1. Pathogen cell wall proteins (CWPs) play important roles during infection (De Groot *et al.*, 2004). For example, they can act as a barrier that can protect against the defense reaction from the host, and further they contribute to the adhesion to host tissues making them important determinants for pathogenesis (Sundstrom, 2002; De Groot *et al.*, 2004). Sometimes, fungal cell wall components are targets for the development of fungicides against the pathogen (Fernandez Acero *et al.*, 2011). Relative to *R. commune* cell wall, work done by Pettolino *et al.* (2009) showed that *R. commune* cell wall is mostly composed of (1,3/1,6)-beta-D-glucans, (1,3;1,4)-beta-D-glucans, galactomannans, rhamnomannans and chitin. We aimed to characterize proteins that are likely to contribute to *R. commune* fitness and virulence. *Rsu3_07158* characterization should provide some insights into the importance of this protein for the structure and composition of *R. commune* cell wall and its pathogenicity on barley.

The yeast *S. cerevisiae* has been widely adopted as one of the best species to carry out homologous recombination (Joska *et al.*, 2014), and it was the one chosen in our lab for the generation of gene replacement constructs for the *R. commune* candidate gene *Rc2* and *Rsu3_07158*. Gene knockout in *R. commune* is challenging but for the fact it is a haploid organism, the procedure should be feasible. Moreover, successful gene deletions have previously been carried out for NIP1, NIP2 and NIP3 (Kirsten *et al.*, 2012).

This study aimed for the characterisation of molecules released by *R. commune* during the interaction with its host barley. Analyses of transcripts produced by *R. commune* during infection of barley plants are a valuable resource for the identification of candidate pathogenicity factors. In this study, we report the identification of two new secreted proteins named Rc2 and Rsu3_07158. Infiltrations with Rc2 protein produced using *P. pastoris* did not trigger cell death in dicot or monocot species tested. Virulence testing through detached leaf assay revealed that the virulence shown by the *R. commune* strains AU2, L77 and L2A in

the barley line SLB-10-009 is not correlated with amino acid changes in Rc2 protein sequences. Unfortunately our efforts to characterise Rc2 and Rsu3_07158 from *R. commune* as novel secreted proteins through targeted gene knockout were not successful.

6.2 Results

6.2.1 *Rc2* gene from *R. commune* has two allelic forms

Mapping of sequence reads for an additional seven sequenced *R. commune* strains against genome sequence of *R. commune* strain 13-13 indicated the presence of the *Rc2* candidate effector gene in all of them. Rc2 is a secreted protein of 74 amino acids with 4 cysteine residues (Fig. 6.2b). Sequence analysis of this gene also revealed the presence of two distinct SNPs (Fig. 6.2a). Both SNPs led to nonsynonymous substitutions suggesting that this gene is under diversifying selection. Four out of eight tested strains carry the Rc2-LQ allele, including UK strain 13-13 and L2A, and the Rc2-VR allele for the remaining 4 strains, including Australian isolate AU2. Four strains including AU2 and 214 had a SNP at position 97, leading to a change in amino acid from valine to leucine and another SNP at position 188, leading to a amino acid change from arginine to glutamine (Fig. 6.2a,b). BLAST search indicated that Rc2 protein does not share homology with any other fungal proteins.

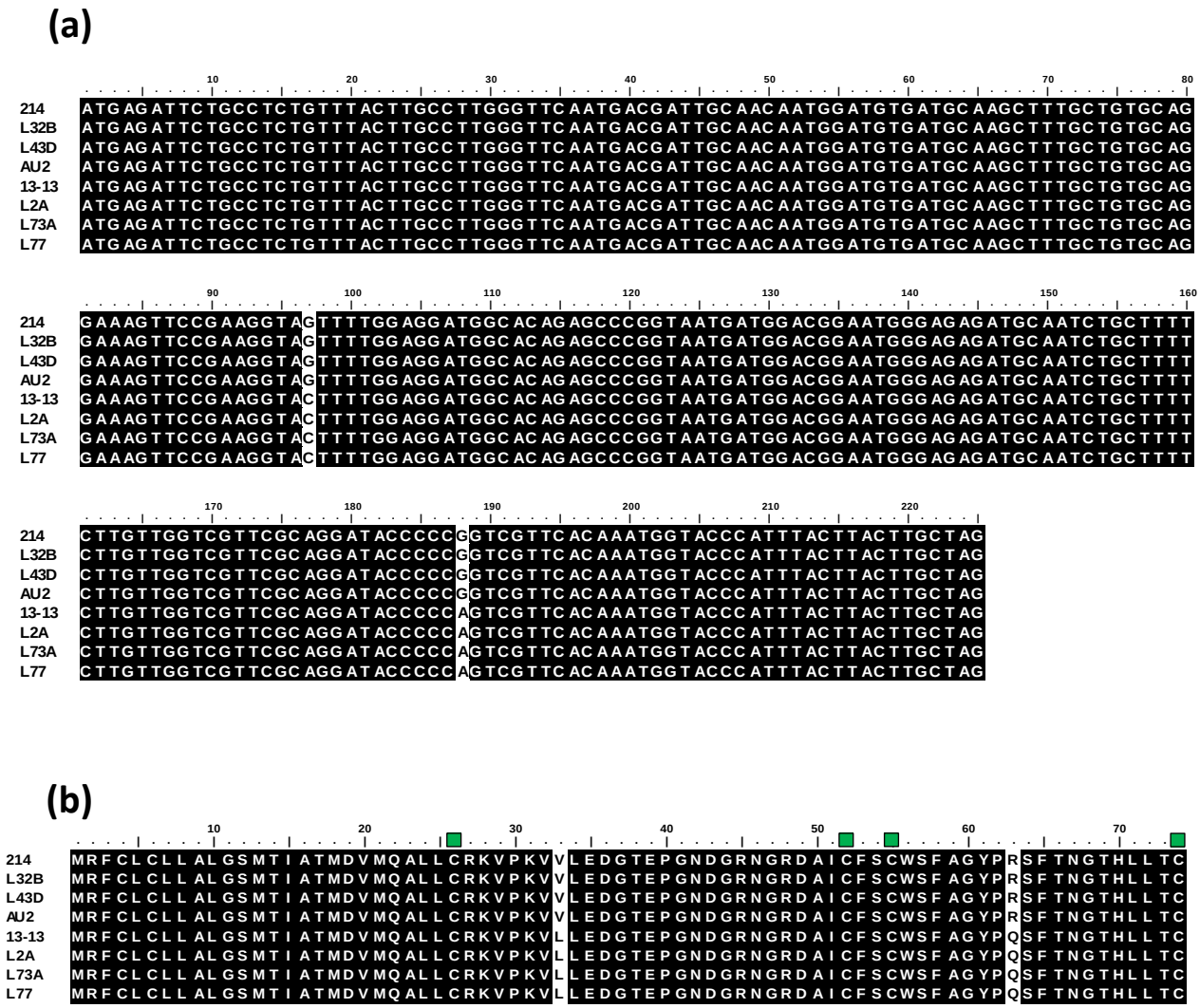

Figure 6.2 ClustalW alignment of DNA sequences and protein sequences of *R. commune* Rc2. (a) ClustalW alignment of DNA sequences of *R. commune Rc2* gene from eight strains showing 2 single nucleotide polymorphisms (SNPs) at positions 97 and 188. (b) ClustalW alignment of amino acid sequences of *R. commune* Rc2 protein from eight strains showing the two changes in amino acids due the non-synonymous SNPs found within *Rc2* coding sequence. Green squares indicate the conserved cysteine residues.

6.2.2 Virulence testing of *R. commune* strains with different alleles of *Rc2*

Around 60 barley genotypes showing high levels of resistance to *R. commune* over 3 years of testing in the field were screened for recognition of individual *R. commune* effectors transiently expressed using BSMV-based expression system. Initially necrotic and chlorotic lesions were shown in barley line SLB 10-009, one of the most resistant lines evaluated, after expression of LQ allele of *Rc2*. This result suggested that an unknown major resistance gene is able to recognize candidate effector Rc2 (Fig. 6.1). To investigate if there is a correlation

between the *Rc2* alleles (VR and LQ) and the virulence of the three sequenced *R. commune* strains, AU2 carrying the *Rc2-VR* allele and strains L77 and L2A carrying the allele *Rc2-LQ,* were tested for their ability to cause symptoms on barley leaves of susceptible cv Optic and line SLB 10-009 using the detached leaf assay. Leaf tissue was evaluated macroscopically for the presence or absence of lesions due to fungal growth during *R. commune* infection. Lesion size was very similar at the time points of 13 and 17 dpi, so 17 dpi was used for evaluation.

In both experiments, strains AU2, L77 and L2A caused symptoms on both susceptible cv Optic and the line SLB 10-009 (Fig. 6.3a,b,c,d,e). The detached leaf assay also showed that at the time of the test strain L77 was the most aggressive of the 3 *R. commune* strains tested causing the biggest lesions on susceptible cv Optic, while strain L2A was shown to be the least aggressive.

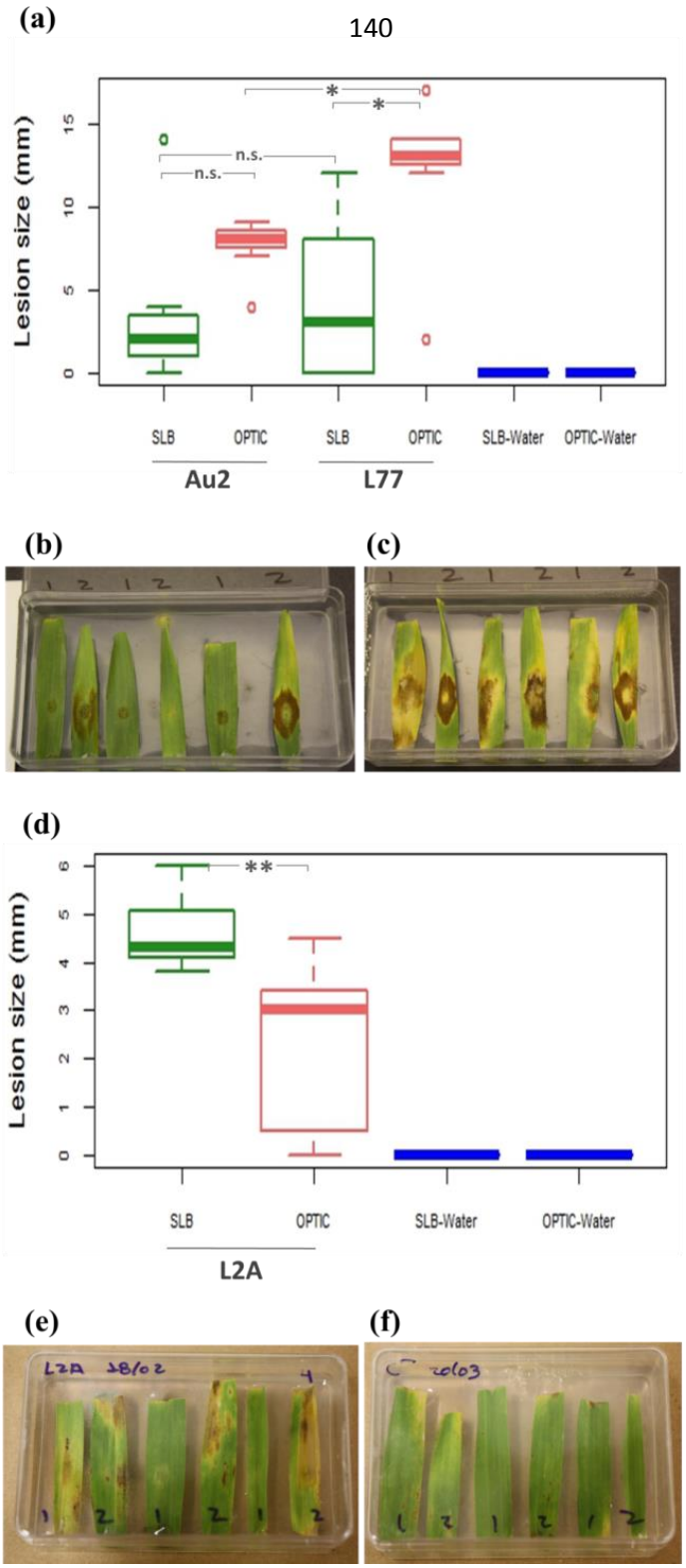

Figure 6.3. Virulence of *R. commune* strains AU2, L77 and L2A on barley line SLB 10-009 and susceptible cv Optic does not correlate with *Rc2* allele distribution in these strains. (a) Size of lesions, in mm, caused by strains AU2 and L77 in leaves of line SLB 10-009 and susceptible cv Optic. Inoculations with water were used as negative control. (b) Lesions caused by strain AU2. (c) Lesions caused by strain L77. (d) Size of the lesions caused by strain L2A in line SLB 10-009 and susceptible cv Optic. Inoculations with water were used as negative control (e) Lesions caused by strain L2A. (f) water control. 1- barley line SLB 10-009, 2- susceptible cv Optic. Data are presented as boxplots with the corresponding medians and 25th and 75th percentiles. *P < 0.05; **P < 0.01; n.s. - not significant.

6.2.3 Rc2 protein does not induce cell death in barley line SLB 10-009 with resistance to *R. commune* or other monocot and dicot plants

To further investigate whether LQ allele of Rc2 is recognised by the barley line SLB 10-009, *P. pastoris* CS containing Rc2-LQ tagged with V5 and V5 tag alone were infiltrated in barley lines SLB 10-009 and Optic. No cell death was observed in leaves from both tested lines after infiltration with Rc2-LQ or V5 tag alone used as a control. LQ allele was initially suggested to be the avirulent allele involved in the putative recognition of Rc2, according to the cell death observed in barley line SLB 10-009 after expression of *Rc2-LQ* allele using BSMV-expression system. Therefore, results obtained with BSMV system do not agree with the results obtained with *P. pastoris* expression system. Thus, additional experiments are required to confirm *Rc2-LQ* as the avirulent allele of the protein Rc2 and also to test for the presence of a resistance gene in the barley line SLB 10-009 involved in this potential recognition.

In order to confirm that the cell death triggered by RcCDI1 using *P. pastoris* CS containing RcCDI1-V5 protein in *N. benthamiana* was specific to this protein, Rc2 was used as a control protein for being another apoplastic effector from *R. commune* (Chapter 3). Infiltration of *P. pastoris* CS containing LQ allele of Rc2-V5 protein into the apoplastic space of *N. benthamiana* leaves and CS of *P. pastoris* expressing a V5 tag alone did not induce plant cell death (Fig. 3.4). Here, we also infiltrated *P. pastoris* CS containing Rc2-V5 into leaves of several plant species. Rc2-V5 did not induce cell death in any of the dicot and monocot species tested, including *N. sylvestris*, tomato (*S. lycopersicum*) cv Moneymaker, pepper (*Capsicum annuum*), oilseed rape (*Brassica napus*) cv Mascot or monocots including barley (*H. vulgare*) cv Optic and line SLB 10-009, wheat (*T. aestivum*) cv Tybalt, rye (*S. cereale*), or maize (*Z. mays*) cv Golden Jubilee (Figure 6.4). Infiltrated areas exposed to UV light excitation did not show any fluorescence associated to cell death, suggesting that there was no accumulation of phenolic compounds. Little fluorescence observed in tomato, barley and rye leaves is due to wounding and *P. pastoris* CS remaining on the leaf surface after infiltration.

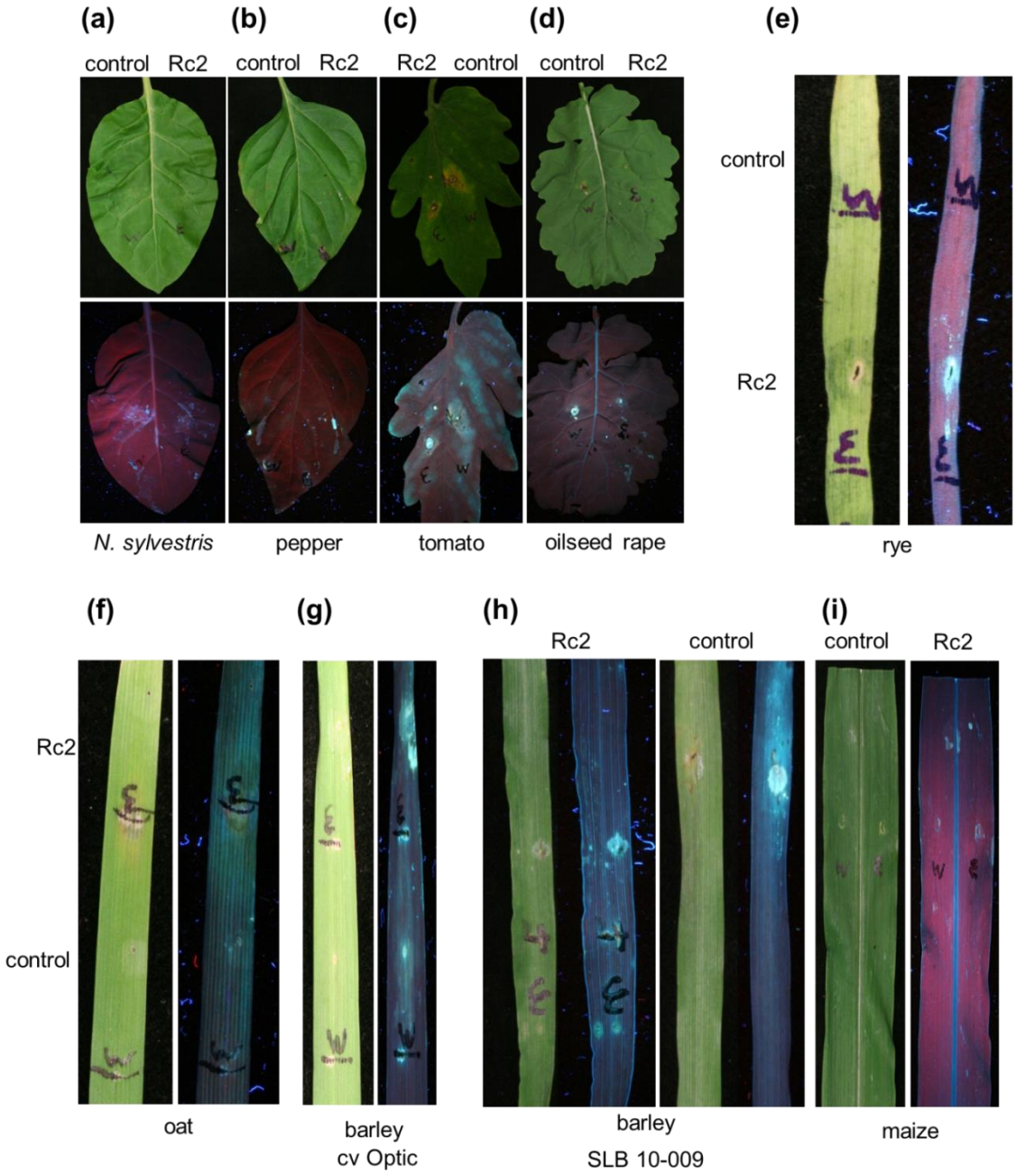

Figure 6.4 Rc2 protein produced by *P. pastoris* does not induce cell death in the dicot and monocot species tested. All plant species were infiltrated with *P. pastoris* culture supernatant (CS) containing Rc2-V5 or *P. pastoris* CS containing V5 tag alone as a control. Representative leaves of (a) *N. sylvestris*, (b) pepper, (c) tomato, (d) oilseed rape, (e) rye, (f) oat, (g) barley, cv Optic (h) barley line SLB 10-009, (i) maize. Photos were taken at 8 days post infiltration.

6.2.4 *Rsu3_07158* sequence analysis

Blast searches of the NCBI database with the Rsu3_07158 predicted protein sequence revealed a match to predicted proteins, similar to hydrophobic surface binding protein A, (HsbA) from *B. cinerea* (1e-40), *Aschersonia aleyrodis* (6e-29), *Ustilaginoidea virens* (1e-25), *Metarhizium guizhouense* (1e-21), *Metarhizium rileyi* (2e-19), and *Zymoseptoria brevis*

(3e-13), which represent fungal species pathogenic on plants and insect (Fig. 6.5, Table 6.1). The finding of the match to these HsbA domain-containing proteins provides some insights into the possible function of the *R. commune* putative protein Rsu3_07158 as a surface-active protein promoting the degradation of hydrophobic solid materials. The protein sequence alignment of *R. commune* Rsu3_07158 with its homologues from other fungal species showed some conserved regions besides the HsbA domain running from amino acid 25 to amino acid 138 (grey-filled box) (Fig. 6.5).

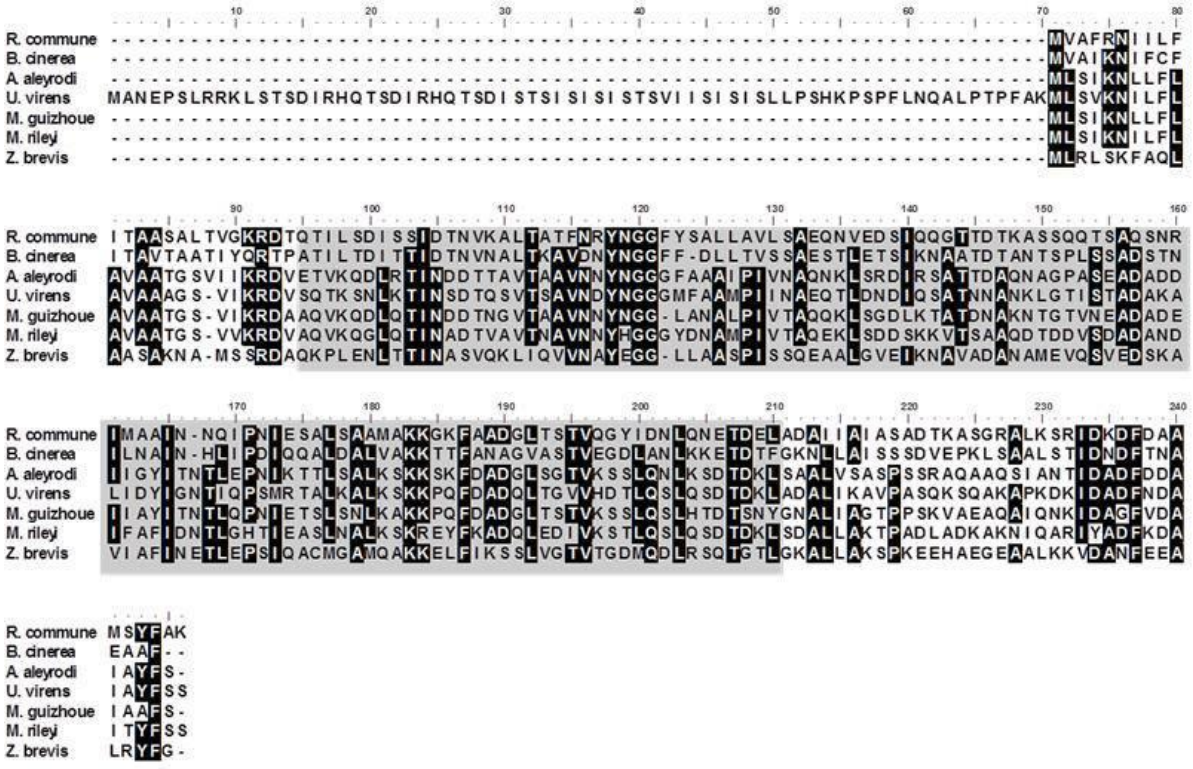

Figure 6.5 ClustalW alignments of protein sequences of *R. commune* putative HsbA domain-containing protein Rsu3_07158 with its homologues from other fungal species. *B. cinerea* T4 (CCD43757.1); *U. virens* (KDB18259.1); *M. guizhouense* ARSEF 977 (KID86769.1); *A. aleyrodis* RCEF 2490 (KZZ99647.1); *M. rileyi* RCEF 4871 (OAA35437.1); *Z. brevis* (KJX93623.1). HsbA domain present from amino acid 25 to amino acid 138 is highlighted in grey.

Table 6.1. *R. commune* HsbA domain-containing protein Rsu3_07158 homologues from different fungi.

Fungal species	Accession number	E value	% similar amino acids	% identical amino acids	Host species
Botrytis cinerea T4	CCD43757.1	1e-40	63%	47%	multiple dicot species
Ustilaginoidea virens	KDB18259.1	1e-25	44%	24%	Rice
Metarhizium guizhouense ARSEF 977	KID86769.1	1e-21	58%	33%	Insects
Aschersonia aleyrodis RCEF 2490	KZZ99647.1	6e-29	63%	33%	Insects
Metarhizium rileyi RCEF 4871	OAA35437.1	2e-19	59%	30%	Insects
Zymoseptoria brevis	KJX93623.1	3e-13	48%	27%	Barley

6.2.5 An attempt to obtain *Rc2* and *Rsu3_07158* knockout transformants

As described in chapter 2, the deletion cassette for *Rc2* and *Rsu3_07158* were produced by fusing the upstream and downstream regions of each gene to the selection marker hygromycin (hph) and the linearized vector PRS426 in *S. cerevisae* by homologous recombination (Fig. 2.1). The deletion cassette was then amplified by PCR in two overlapping fragments, both of them were used together to transform *R. commune* (Fig. 6.6a,b). For the correct integration of the deletion cassette and the replacement of the gene of interest, three recombination events should occur, two between the flaking regions and the genomic DNA and one for the hygromycin gene. The transformation procedure was successful as indicated by the fact that *R. commune* colonies were growing on the media with the selection marker (hygromycin) (Fig. 6.6d). In the case of *Rc2*, the colonies obtained got contaminated so they could not be screened. Similar to the procedure done for *RcCDI1* gene knockout, DNA extraction was performed for each of the transformants for *Rsu3_07158* gene and they were screened by PCR. Amplifications of the wild type size PCR product were obtained for left and right flaking regions using the first (WTFL, WTRL) and the second (WTFR, WTRR) primer sets respectively, and also for the hygromycin gene using the third primer set (hphF, hphR) (Fig.

6.6c). No amplification was obtained when checking for the insertion of the hygromycin gene in the expected genome location in order to knockout the gene of interest, using the third primer set (WTFL, HPHR). 87 colonies were screened for *Rsu3_07158*, but no successful transformants were obtained. Actin band was amplified for all samples as a positive control (Fig. 6.6c).

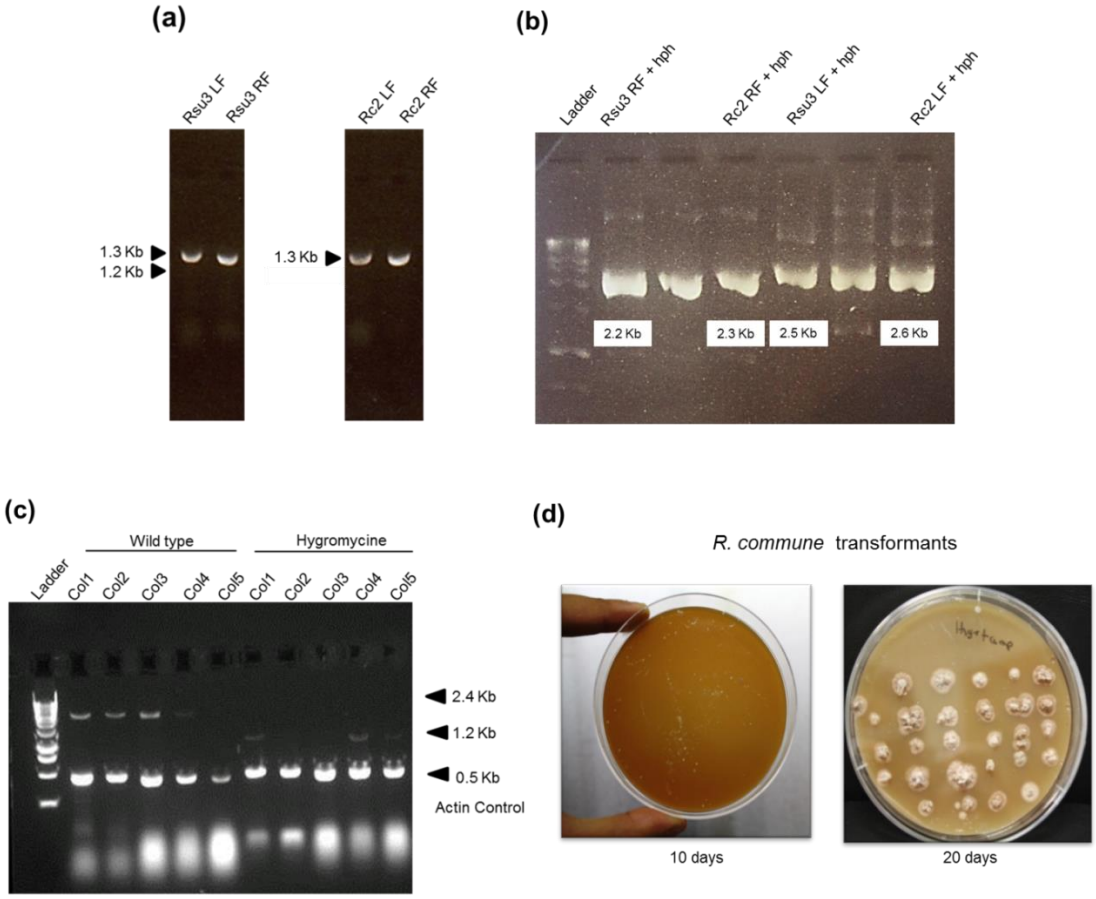

Figure 6.6 Knockout strategy for *R. commune* candidate genes *Rc2* and *Rsu3_07158* using yeast homologues recombination system. (a) PCR products for the right and left flaking regions of the genes *Rc2* and *Rsu3_07158* (b) Amplification of the deletion cassette as split marker strategy-PCR products corresponding to the left and right flanking regions for each gene fused to a part of the hygromycine (hph) gene previously assembled using yeast homologous recombination. The products were then used to transform *R. commune* by electroporation. (c) Screening of *R. commune* transformants for *Rsu3_07158* gene disruption. Agarose gel showing the amplification of the left flanking region for the wild type gene *Rsu3_07158* in five colonies, and the presence of the full length hygromycine gene. Amplification of Actin gene was used as a positive control. (d) *R. commune* growing on plates with selection medium (hygromycine) 10-20 days after transformation by electroporation.

6.3 Discussion

Control of leaf scald is not very effective because *R. commune* is a very variable pathogen rapidly overcoming fungicides and deployed barley genetic resistances (Avrova & Knogge, 2012). Our efforts are focused on the characterisation of potential pathogen effectors from *R.* commune that will hopefully help in the identification of important immunity processes targeted by *R. commune* in plants, and thereby ultimately help with breeding new resistant cultivars. So far, a small family of necrosis inducing peptides (NIPs) have been identified (Wevelsiep *et al.*, 1991) and *R* genes acting against *R. commune* have been previously described (Bjørnstad *et al.*, 2002), but because little is known about this plant-pathogen interaction, it is necessary to concentrate our research efforts on the understanding of other *R. commune* effectors and their targets, the majority of which are still unknown. Sequencing of RNA from barley leaves infected with *R. commune*, led to the identification of *Rc2*, encoding a 74 amino acid secreted protein with 4 cysteines, and *Rsu3_07158*, encoding a putative hydrophobic surface binding protein A.

qRT-PCR has been one of the best strategies to characterize fungal development *in planta* (Gachon & Saindrenan, 2004). This methodology has been used before to characterize the expression of *R. commune NIP*s during infection process in barley (Kirsten *et al.*, 2012). Similar to *NIPs* and *RcCDI1*, *Rc2* and Rsu3_07158 genes were upregulated early during infection which builds a correlation between this early gene expression and the first stage of infection by *R. commune* in barley leaves. At this stage, fungal conidia are germinating on the leaf surface and penetrating the leaf cuticle, establishing fungal growth between cuticle and epidermis. This is associated to the biotrophic phase of the fungus (Zhan *et al.*, 2008; Avrova & Knogge, 2012).

Rc2 sequences were found in all eight *R. commune* strains evaluated; multiple alignment of DNA sequences of *Rc2*, showed the presence of only two SNPs between all of them. These

two SNPs led to nonsynonymous substitutions, suggesting that the action of Darwinian or positive selection has been leading the changes of this gene sequence, but it is uncertain whether these changes affect the still unknown function of the protein. *R. commune* strain AU2 carries the allele *Rc2-VR*, while strains L77 and L2A, carry the allele *Rc2-LQ*. Preliminary screen of around 60 barley genotypes that showed high levels of resistance to *R. commune* in the field, with BSMV expressing L2A allele of candidate effector *Rc2* suggested that barley line SLB-10-009 might be able to recognise this allele and that Rc2 might be an avirulence protein. To test this hypothesis we used detached leaf assay on barley leaves of susceptible cv Optic and line SLB 10-009 to look for correlation between *Rc2* alleles with virulence, and if correlation was found, to define *Rc2* as a potential avirulence gene triggering resistance in a line via as yet unknown *R* gene.

In a first experiment, strains AU2 and L77 caused lesions in both susceptible cv Optic and line SLB 10-009, indicating that both strains carrying different alleles (*AU2-VR, L77-LQ*) were virulent on line SLB 10-009. Similarly, in a second experiment, lesions were obtained for the line SLB 10-009 and cv Optic following inoculation with the strain L2A indicating the presence of the virulent allele. In conclusion, *R. commune* strains AU2, L77 and L2A carrying alternative alleles of *Rc2* gene were tested but no correlation was found between genotype and phenotype, the virulent and avirulent alleles could not be identified.

The results obtained here showed that detached leaf assay is a good method to evaluate resistance to *R. commune*. As it is a hemibiotrophic fungus (Oliver & Ipcho, 2004), even when leaf senescence started with chlorotic and necrotic spots observed, *R. commune* was still able to proliferate and keep infecting. At the moment not much is known about the type of resistance used by this barley line to avoid fungal establishment or the mechanisms used by the fungus to overcome resistance and establish a successful infection.

Effectors are essential molecules for pathogenicity (Thomma *et al.*, 2011). To test if Rc2 is acting as a pathogenicity factor, further studies need to be carried out. Barley plants could be

inoculated with *R. commune* after infiltration with this protein to see if it enhances fungal infection and colonisation. Similar results, have been achieved for some of the effectors studied up to now; some examples are the RXLR effectors from the potato pathogen *P. infestans* Pi04314, AVR1, PexRD2 and AVRblb2 (Bozkurt *et al.*, 2011; King *et al.*, 2014; Du *et al.*, 2015; Boevink *et al.*, 2016). In the same way, blast searches indicated that Rc2 protein sequence did not share homology with any other fungal proteins so it seems to be exclusive to the *R. commune* genome, which is a common characteristic of pathogen effectors. Infiltrations with Rc2 protein produced by *P. pastoris* did not induce cell death response in any of the barley lines tested, suggesting the absence of any direct recognition of Rc2 in barley line SLB 10-009. No cell death response was also caused by Rc2 protein in any other monocot or dicot species tested making it a perfect negative control for RcCDI1.

In the case of the gene *Rsu3_07158*, BLAST searches provided information of the presence of some homologues in other fungal species, encoding proteins with hydrophobic Surface Binding Protein A (HsbA) domain. In *Aspergillus oryze* the surface binding protein HsbA, was found to be secreted into the culture medium, place where it was isolated from for further analysis (Ohtaki *et al.*, 2006). It was shown to promote the degradation of polybutylene succinate-co-adipate (PBSA) hydrophobic surface by the use of the cutinase *CutL1* (Ohtaki *et al.*, 2006). PBSA is very similar to the wax polymers found in plants, as in the leaf cuticle, for example, suggesting that HsbA proteins may be important for the pathogen during the infection process. These findings indicate the potential role for *R. commune* Rsu3_07158 as a HsbA containing protein during the first stage of infection which involves the cuticle penetration. Similarly, six genes encoding proteins with HsbA domains have been identified in a transcriptome analysis of *Mycosphaerella fijiensis* during infection in banana (Noar & Daub, 2016).

We also aimed to elucidate *Rc2* and *Rsu3_07158* gene function by obtaining knockout transformants using the homologous recombination repair pathway (Clikeman *et al.*, 2001).

Colonies growing on selection medium were obtained after *R. commune* transformation by electroporation. Colonies for *Rc2* gene were contaminated and could not be evaluated. Colonies for the *Rsu3_07158* gene were screened and amplification of wild type and hygromycine gene was obtained but no amplification took place when checking for the replacement of the gene of interest by the selection marker (hph) due to the integration of the deletion cassette in the expected genome location. Therefore gene knockouts could not be achieved for any of the genes tested. The recombination efficiency for many filamentous fungi is very low (Paietta & Marzluf, 1985), so it is highly recommended to use the strain deficient in the NHEJ repair pathway, to increase the levels of homologous recombination and hence facilitate the generation of *R. commune* gene knockouts.

In summary, sequencing of the interaction transcriptome from an early time point during infection of barley leaves with *R. commune* led to the identification of two candidate proteins, Rc2 and Rsu3_07158. Multiple alignment of Rc2 protein sequences of eight *R. commune* isolates, infiltration of Rc2 protein produced by *P. pastoris* into leaves of several dicot and monocot species and virulence testing were used to characterise this protein and elucidate its role as a potential pathogenicity factor. Attempts to obtain *Rc2* and *Rsu3_07158* gene knockouts were not successful. An improved method is required in order to achieve a knockout for both genes tested. Host resistance is the most suitable method to protect barley from *R. commune* (Avrova & Knogge, 2012), but we also must ensure that such resistance found is durable. To achieve this, it is required to gain a broad understanding of the biology of *R. commune*. Therefore, unveiling the role of *R. commune* proteins secreted during infection will be useful in developing management strategies against this devastating pathogen.

Chapter 7. Overall Discussion

7.1 Global food demand security

Global food demand is rising and food production needs to increase. Agriculture has been facing many different challenges in the last few years including climate change, extreme weather conditions, loss of agricultural land due to erosion and construction factors, increasing numbers of pests, and crop plant pathogens getting even more resistant to chemical control measures (Bebber *et al.*, 2013; Sundström *et al.*, 2014). We are facing a global food security problem that involves poverty, lack of assistance to small farmers, inadequate food distribution, environmental impact and complex political and economic factors. Immediate action is required from governments and the academic community to intervene and provide the best solution to these problems, which can only be achieved if we all put together our efforts and work as a unit.

Plant pests and pathogens continuously affect crop productivity (Oerke, 2006). Integrated systems of agricultural production need to be implemented to achieve an effective control of these biotic stresses. There are many healthy farm practices to protect crops, which include crop rotation, especially for non-host crops to stop the spread of pests or diseases (Sumner, 1982; Hwang *et al.*, 2015). Soil and water conservation practices such as tillage (Busari *et al.*, 2015) and several agricultural activities, including clean seeds (Zhan *et al.*, 2008), planting density (Dinoor & Eshed, 1984) and plant breeding (Brown & Caligari, 2008) also help in controlling diseases. Pesticide applications have been widely used for decades to protect crops from pests and diseases. It is calculated that 5.6 billion pounds of pesticides are used worldwide (Alavanja, 2009).With a lack of good management and training programmes, pathogens become resistant to these chemical products, as is the case for many different fungal and oomycete pathogens (Bollen & Scholten, 1971; Taggart *et al.*, 1998; Parra & Ristaino, 2001; Hausbeck & Lamour, 2004).

Biotechnological techniques have been extremely useful in efforts to enhance plant resistance to pests and pathogens. The use of genetically modified (GM) crops has been shown to be promising when aiming to improve plant disease resistance (Shah *et al.*, 1995). To generate GM crops, single or multiple genes are introduced or altered in the desired crop by genetic modification to alter or introduce a specific desired trait (Whitman, 1999). At the present, these crops are completely not accepted by the society; the major concerns involving their putatively harmful environmental impact and their possible risk to human health (Conner *et al.*, 2003); however, many achievements have been made and the use of GM crops is still considered a promising strategy for disease control.

7.2 Integrated crop protection system against *R. commune*

R. commune, as the causal agent of barley leaf scald, has major economic impacts in the UK. This pathogen is estimated to cause yield losses of £10.8 million per annum (at a price of £225/tonne) after fungicide treatment (King *et al.*, 2013). *R. commune* disease control relies on chemical applications but it is able to evolve quickly (Zhan *et al.*, 2008), for this reason the use of different fungicide groups is required according to their specific mode of action. Also, the use of resistant barley cultivars and implementation of effective cultural practices are recommended (Avrova & Knogge, 2012). Constant efforts have been made in the use of these agricultural practices, but they have been proven to not be completely effective. Therefore, the understanding of the molecular mechanisms used by *R. commune* to infect barley will hopefully help in finding more durable resistances to this pathogen.

7.3 *R. commune* transcriptome sequencing reveals high abundance of transcripts with unknown function named *RcCDI1, Rc2* and R*su3_07158*

The analysis of transcriptomes has become a strong and effective method used to detect a set of candidate genes turned on and off in a single cell or tissue, inferring genes involved in specific biological processes, and how these transcriptional changes are correlated with the

increase or reduction of disease states (Imam *et al.*, 2016). Recent sequencing of RNA from barley leaves infected with *R. commune* (Penselin *et al.*, 2016) has led to the identification of a large number of candidate genes with potential roles in virulence. It increases our knowledge of several key aspects of infection by *R. commune* at molecular level and thus directs our research on the characterisation of these genes, for a better understanding of the pathogenicity mechanisms involved in this plant-pathogen interaction. Many biologically relevant genes can be identified in a transcriptome analysis, but our research efforts are directed towards the identification of plant pathogen effectors as one of the most widely studied groups of genes involved in pathogenicity processes (Bhat & Shahnaz, 2014). We started by conducting a search of candidate genes with the characteristics associated to candidate effector proteins in the *R. commune* secretome. The selection criteria was based on the following characteristics: (a) proteins of small size (less than 200 aas), (b) cysteine rich proteins, (c) expression during infection, (d) species-specific (Jones & Dangl, 2006; Stergiopoulos & de Wit, 2009; Sperschneider *et al.*, 2015; Thatcher *et al.*, 2016). Candidate effector proteins meeting these features have been identified in a series of fungal pathogens, such as *M. oryzae* (Kim *et al.*, 2010), *B. graminis* f. sp. *hordei* (Schmidt *et al.*, 2014), *F. oxysporum* f. sp. *lycopersici* (Lievens *et al.*, 2009), and *M. lini* (Nemri *et al.*, 2014).

Guided by these criteria, candidate genes initially named *Rc1* (*R. commune* 1), *Rc2* (*R. commune* 2) and *Rsu3_07158* were identified. They are small secreted proteins, Rc1 and Rc2 protein sequences with four cysteine residues and highly expressed early during infection. At this stage of infection, the fungus is penetrating leaf cuticle and establishing infection in the apoplastic space of its host barley (Wevelsiep *et al.*, 1991). In addition to this information and in collaboration with researchers from Rothamsted Research, it was found that Rc1 induced cell death in *N. benthamiana* when it was overexpressed using a BSMV-based expression system. It was renamed to *RcCDI1* (*R. commune* Cell Death Inducing 1). Using the same BSMV system, it was initially shown that necrotic lesions were triggered by the

overexpression of Rc2 in the barley line SLB 10-009, which is a line that showed high level of resistance in the field. This suggested that Rc2 could be potentially recognised in the barley line by the presence of a still unknown major resistant gene. At this stage, nothing was known about the function of the genes *RcCDI1* or *Rc2*. In contrast, sequence similarity searches with NCBI BLAST and InterProScan, indicated that the gene *Rsu3_07158* encoded for a hydrophobic surface binding protein. Due to all these characteristics, these three genes were considered as good candidates for further characterization at the molecular level.

7.4 RcCDI1 identification as a novel fungal PAMP

PAMPs perception by PRRs leads to the activation of a layer of plant defence responses referred to as PTI (Medzhitov & Janeway, 1997; Jones & Dangl, 2006; Nicaise *et al.*, 2009). The classical features that define a PAMP are: (a) wide conservation between microbial species, (b) recognition by a broad host range of plant species, for the fact that PRRs are widely conserved, (c) molecules difficult for pathogen to alter because they are essential for microbial survival, and (d) recognition by PRRs (Jones & Dangl, 2006; Thomma *et al.*, 2011). Based on this definition, the results obtained from the characterisation of RcCDI1 support our hypothesis that this protein is a PAMP, and the evidence for this is explained as follows.

R. commune RcCDI1 protein and its homologues from other ascomycete species including *Z. tritici*, *M. oryzae*, *B. cinerea*, *S. sclerotiorum*, and *N. crassa* were found to induce a strong cell death in *N. benthamiana*, clear evidence of RcCDI1 conservation within the ascomycete group. The fact that *RcCDI1* homologue from *B. graminis* (BgCDI1) did not trigger cell death in *N. benthamiana*, is an open door to investigate its structure and establish a differentiation from those homologous protein sequences triggering cell death. Moreover, it will give us insights into the possible protein sequence involved in the recognition events of RcCDI1 or a possible way of avoiding recognition by BgCDI1 in the plant.

It has been previously stated that ETI is a more prolonged and robust response than the responses of PTI and it is typically associated with an HR (Tsuda & Katagiri, 2010). The RcCDI1-induced cell death is explained by the fact that ETI and PTI can share some signalling components; and even signalling cascade leading to HR for some cases. Several examples of HR induced by PAMPs include the glycoprotein CBEL from *P. parasitica* var. *nicotianae* in tobacco and Arabidopsis (Khatib *et al.*, 2004), the elicitor harpin from *Erwinia amylovora* in tobacco (Wei *et al.*, 1992), elicitin INF1 from *P. infestans* in plants of *Nicotiana* spp. (Kamoun, 1998), and the bacterial flagellin peptide flg22 from *P. syringae* in Arabidopsis (Naito *et al.*, 2007).

RcCDI1 gene was present in all 32 *R. commune* isolates tested. From the 6 SNPs found between the strains, just 2 led to nonsynonymous substitutions, which is a clear evidence of the conserved structure of *RcCDI1*. In addition, RcCDI1 produced by *P. pastoris* also triggered cell death in *N. sylvestris*, tomato and potato plant species, all of them part of the Solanaceae family. No cell death was observed in any other dicot and monocot species tested, including the host plant barley. This last finding did not challenge our hypothesis of RcCDI1 being a PAMP because it has been previously shown that the recognition of very well-known PAMPs is achieved by just a specific range of plants (Felix & Boller, 2003; Kunze *et al.*, 2004). It is the case for CSPs from bacterium *S. aureus*, only recognized by plants of the Solanaceae family, and the bacterial PAMP EF-Tu, which appears to be specifically recognized by plants of the Brassicaceae family but not Solanaceae (Felix & Boller, 2003; Kunze *et al.*, 2004). It is also important to mention that the full length RcCDI1 induced cell death in *N. benthamiana* and in contrast, cell death did not happen once RcCDI1 was expressed lacking its signal peptide. This finding indicates that RcCDI1 recognition only takes place when the protein enters the secretory pathway of the cell, being subsequently secreted to the apoplastic space where it is recognised by a given cell surface PRR receptor.

In a search for the molecular mechanisms involved in RcCDI1 recognition in *N. benthamiana*, we decided to investigate the role of three key immune components of PAMP signalling (BAK1, SOBIR1 and SGT1). BAK1 has been previously shown to act as a central regulator of plant immunity, with an essential role in PRR-dependent signalling (Chinchilla *et al.*, 2007). SOBIR1 was shown to regulate many RLPs, involved in innate immunity (Liebrand *et al.*, 2013, 2014) and SGT1, as a defence-related gene has been shown to regulate plant cell death responses (Peart *et al.*, 2002). Our results showed that silencing of BAK1, SOBIR1 and SGT1 in *N. benthamiana* suppressed cell death triggered by RcCDI1, demonstrating their requirement for the plant defence response induced after the perception of this novel PAMP. In addition the lack of suppression of RcCDI1-mediated cell death by the co-expression of RcCDI1 with several *P. infestans* RXLR effectors including PiAvr3aKI, PexRD2, PIGT_13628 (PexRD27) and the avirulence gene AvrPto, from *P. syringae* pv. tomato gave us some insights into the potential signalling pathways activated upon recognition of RcCDI1.

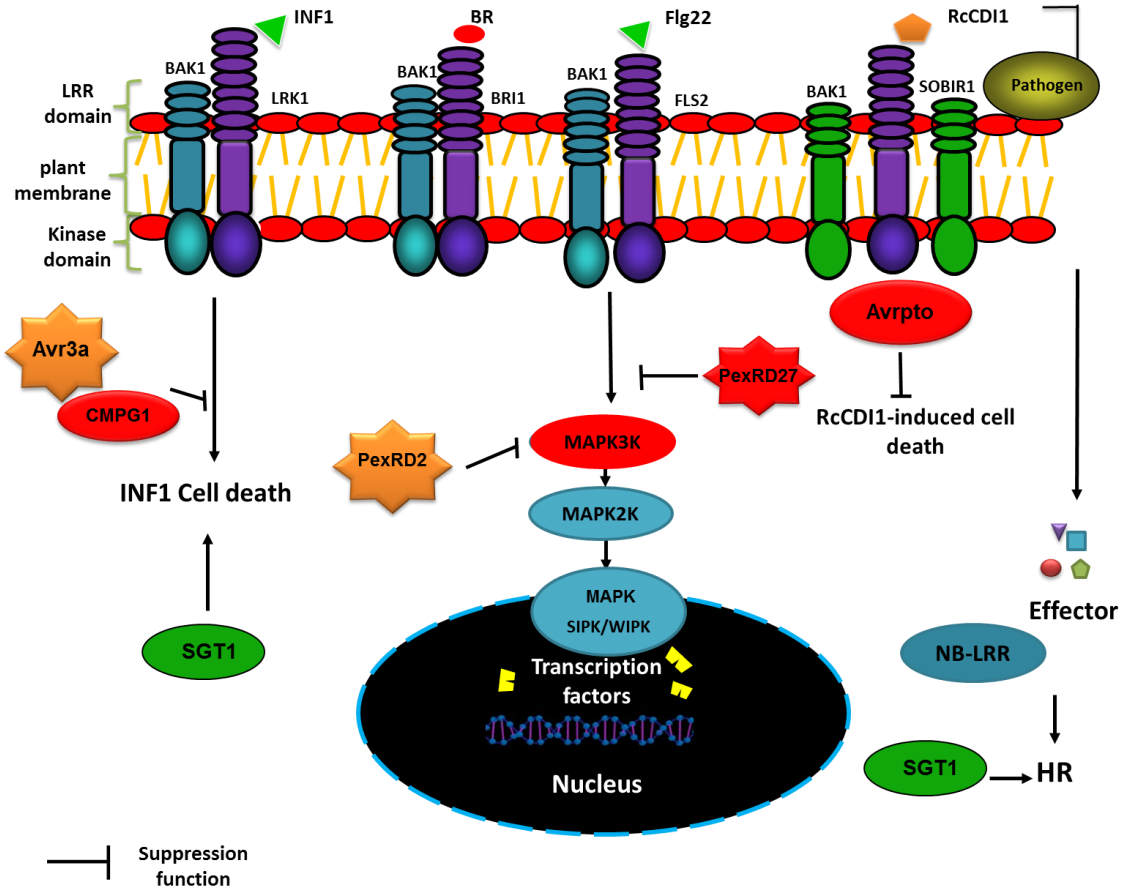

Figure 7.1 Signalling pathways involved in RcCDI1 recognition. RcCDI1-triggered cell death is NbBAK1, NbSOBIR1 and NbSGT1 dependent (Green). In contrast, RcDI1-induced cell death was not suppressed by *P. infestans* RxLR effectors Avr3a and PexRD2, suggesting that NbCMPG1 and NbMAPKKKε are not involved in RcCDI1 recognition (red), respectively. In addition, the lack of RcCDI1-cell death suppression by *P. infestans* RxLR effector PexRD27 (red) suggests that RCDI1-mediated MAP kinase cascade activation is different to the one targeted by flg22/FLS2 protein complex. Moreover, *P. syringae* pv. tomato avirulence gene Avrpto did not suppress RcCDI1-triggered cell death (red), suggesting that RcCDI1 activates signalling pathways similar to those triggered by the *P. infestans* elicitin PiINF1.

Further research is needed to identify the downstream immune signalling pathways elicited by the recognition of this fungal PAMP. The acquisition of all the potential knowledge about the molecular mechanisms used by *R. commune* during infection will shed a new light on the understanding of the plant defence responses, and therefore help us in the search for resistances against *R. commune*.

7.5 Up-regulation of PTI marker genes upon infiltration of RcCDI1 into *N. benthamiana*

Plants are under continuous attack by different plant pathogens. Pathogen detection leads to the rapid and effective activation of immune responses to fend off the attack (Jones & Dangl, 2006). This recognition includes a series of defence responses including dramatic changes in the host transcriptome (Atkinson *et al.*, 2013; Sham *et al.*, 2014). Due to this massive transcriptional reprogramming, several genes have been used as PTI markers for the fact that they are highly upregulated during early stages of PAMP recognition, giving us an insight into the defence mechanisms activated upon microorganisms perception. For example, Gust *et al.*, (2007) showed in his work that similar transcriptional changes occur during bacterial flagellin epitope (flg22) and peptidoglycan (PGN) induced recognition events. As shown in previous chapters, RcCDI1 induces cell death in several species of the Solanaceae family. These observations raise the question of whether or not RcCDI1 induces defence-related gene expression, in particular of genes already known to be upregulated during PTI processes. The up regulation of *NbPTI5, NbACRE31, NbWRKY7* and *NbWRKY8* has been previously shown to occur during PTI responses and therefore these genes are considered as PTI marker genes (Hao *et al.*, 2014; Adachi *et al.*, 2015). Thus we assessed if RcCDI1 was capable of inducing these PTI marker genes. As a result, the four studied marker genes were found to be transcriptionally upregulated in *N. benthamiana* after RcCDI1-V5 infiltration. These results further confirm the RcCDI1 involvement in the activation of PAMP-induced defence responses.

7.6 Transcriptional regulation of PTI marker genes upon infiltration of RcCDI1 into barley

As mentioned previously, RcCDI1 induced cell death in plants of the Solanaceae family, and did not in the limited number of monocot species tested. The immune defence responses in

monocots are not as well characterised when compared to the responses in dicot plants (Takahashi *et al.*, 1999). Besides, all the plant species respond in a different way to a specific stimulus, finding variability even within the same group of plants. Therefore it is not surprising that RcCDI1 presence induces distinct defence responses in dicots. The lack of cell death induction in monocots by RcCDI1 can be explained by the lack of RcCDI1 recognition due to the absence of the putative RcCDI1 receptor in monocots. Moreover this absence of RcCDI1 cell death can be explained by a reduced accessibility of this protein to its site of recognition in monocots or by a recognition event that does not lead to cell death. Similarly to RcCDI1, NLPs induce necrosis in dicots but not in monocots (Bailey, 1995) and the bacterial flagellin peptides flg22 and flg15 did not induce defence response in rice (Felix *et al.*, 1999). As for RcCDI1, the reasons for this absence of response in monocots remains undescribed and similar hypothesis were drawn from these examples (Bailey, 1995; Pemberton & Salmond, 2004).

PAMP perception also leads to the upregulation of genes involved in phytohormone biosynthesis such us jasmonic acid (JA) and salicylic acid (SA); for example, PAMP Pep-13 from potato pathogen *Phytophthora sp.* induces the accumulation of both SA and JA in potato (*S. tuberosum*) (Halim *et al.*, 2004). In addition to the induction of PTI marker genes by RcCDI1 in *N. benthamiana*, we wanted to test if similar responses were induced in the host barley even in the absence of cell death after infiltration with RcCDI1. A slight upregulation was shown for marker genes, SA marker, *HvPR1*, and the JA marker, *HvAOS* post infiltration with RcCDI1-V5. It is suggested that the levels of expression for both marker genes are likely to be within normal variation in transcript abundance, so there is no induction of these PTI marker genes by the infiltration of RcCDI1, explained by the lack of RcCDI1 recognition in barley. Our knowledge has a great potential in future efforts to engineer nonhost resistance in monocots by transferring the PRR involved in RcCDI1 recognition from dicots into monocots through conventional breeding or transgenesis as it has been previously shown in several

studies (Song *et al.*, 1995; Lacombe *et al.*, 2010; Fradin *et al.*, 2011). It is also important to note that the transfer of the receptor from dicot to monocot species will help to induce durable resistance against those pathogens of high economic importance for crop plants like rice and wheat such as *M. oryzae* and *Z. tritici.* Similar findings were shown by Holton *et al.*, (2015), where the chimera between PRRs EFR and XA21 from Arabidopsis and rice respectively induce elf18-signalling in Arabidopsis. These findings are crucial to show that responses triggered by PRRs are conserved not only between plant families, but also within the major groups, monocots and dicots, facilitating transference of PRRs between families. Despite the exciting prospect of transferring these PRRs between species, it is worth taking into consideration possible adverse effects of these receptors on the growth and development of monocot species.

The RcCDI1 mediated upregulation of PTI marker genes in *N. benthamiana* suggests that RcCDI1 is a PAMP with influence on defence transcriptional networks. It will be really interesting to do further research and characterise the activation of other signalling responses downstream of this PAMP perception (including ROS, ethylene (ET) induction and callose deposition) that would in principle lead to enhanced plant resistance (Felix *et al.*, 1999; Luna *et al.*, 2011; Couto & Zipfel, 2016).

7.7 Identification of RcCDI1 amino acid region recognised in *N. benthamiana*

Deletion analyses were carried out to detect the protein regions involved in recognition of RcCDI1. A deletion series of RcCDI1 gene sequence was performed and the resulting truncated proteins were expressed in *N. benthamiana* to investigate the RcCDI1 amino acid residues indispensable for defence responses activation. It was shown that cell death induction is enhanced by the co-expression of the N- and C- terminal domains of RcCDI1. In previous work, efforts to find elicitor domains involved in immune responses have been successful. For example, *Xanthomonas campestris* pv. *campestris* flg22 region of flagellin or the first 18

amino acids of *E. coli* EF-Tu were identified as being responsible for the induction of defence responses in *A. thaliana* (Kunze *et al.*, 2004; Sun *et al.*, 2006).

The identification of the RcCDI1 regions involved in recognition provides opportunities for future research. It would help us in the search of RcCDI1 immune receptor *in planta*, and once it is identified, then the single or the multiple binding sites mediating recognition can be clearly determined. Similarly, Dunning *et al.* (2007) determined the specific domain of FLS2 involved in flg22 perception. According to the mutagenesis study, the LRRs 9 to 15 of FLS2 constitute the domain involved in flg22 perception. Once the RcCDI1/PRR complex has been fully characterised we can use different molecular tools to increase, if required, the responsiveness to RcCDI1 by the plant receptor.

7.8 Characterisation of *R. commune* candidate effector Rc2

The main reason to consider Rc2 a good candidate gene to further characterise was the necrosis observed in barley leaves of line SLB 10-009 upon over-expression of Rc2 using the barley stripe mosaic virus (BSMV)-based expression system. These findings suggested that this event could be explained by a potential Rc2 recognition by an unknown resistant protein. No barley R proteins have been cloned to date against *R. commune* infection and the only Avr protein recognition event in barley against *R. commune* effectors characterized to date is the necrosis inducing peptide (NIP1), recognised in the barley cultivar Atlas 46, carrying the resistance gene *Rrs1* (Hahn *et al.*, 1993; Rohe *et al.*, 1995).

Rc2 gene sequence also contains two SNPs leading to nonsynonymous substitutions. As a result the two allelic forms, L-Q for *R. commune* strains L77 and L2A, and V-R for strain AU2, were evaluated through detached leaf assay to try to find a correlation between the two allelic forms of *Rc2* with virulence in the barley line SLB 10-009. Results revealed the ability of all 3 strains to cause symptoms in line SLB 10-009, so it was not possible to find a correlation. Further BSMV-based expression of Rc2 in the barley line SLB 10-009 failed to

induce the specific necrotic response suggesting that the original observation might have been an artefact.

7.9 *RcCDI1, Rc2* and *Rsu3_07158* gene knockouts

Many different strategies have been described to elucidate gene functions; one of them is based on gene knockouts. We were interested to see if the genes *RcCDI1*, *Rc2* and *Rsu3_07158* play a central role in pathogenicity. RcCDI1 behaviour fits into the definition of PAMPs as they are typically invariant in different microbial species and are essential for pathogen survival (Thomma *et al.*, 2011). For this reason, it is possible that *RcCDI1*gene knockout will be non-viable. Besides, it is worth clarifying that some results have shown PAMPs playing a role in pathogenicity; a typical example is the very well-known flagellin from *P. syringae* pv *tabaci* (Naito *et al.*, 2008) and AX21 secreted protein from *X. oryzae* pv. *oryzae* (Lee *et al.*, 2006).

Many transformation procedures have been developed for filamentous fungi (Galagan *et al.*, 2003). Electroporation technique was chosen in our lab and was identified as a highly effective method to transform *R. commune*, despite that no successful knockouts were achieved for *RcCDI11*, *Rc2* or *Rsu3_07158* genes. Recommendations for future research involve: (a) obtain deletion strain for ku70/80 genes to remove non-homologous end joining (NHEJ) repair pathway in filamentous fungi (Choquer *et al.*, 2008; Koh *et al.*, 2014), (b) use gene silencing instead of gene replacement, (c) develop CRISPR-Cas9 constructs for *R. commune* genome editing. Future success of knockout strategies might help to unveil the true functions of these, as well as other proteins that could lead to new insights into *R. commune* biology.

7.10 Future perspectives for *R. commune* candidate genes: new insights into host-pathogen interactions to achieve durable resistance

The effectiveness of controlling the devastating pathogen *R. commune*, is based on the understanding of the molecular mechanisms involved in the interaction between *R. commune* and its host barley. Considering plant resistance as the most effective strategy to combat the attack not only by *R. commune*, but also by other pests and pathogens, our efforts are centralised in the search for durable resistance within an effective control management system. Deeper understanding of the ability of the pathogen to overcome this resistance is hugely linked to the study of effector proteins.

Our results clearly indicate that RcCDI1 is acting as a PAMP. However, questions relative to the specific PRR involved in RcCDI1 recognition, the signal transduction pathways triggered upon RcCDI1detection, and most importantly, the specific role of this novel fungal PAMP in *R. commune* fitness, are still in the process of being resolved. As potential experiment to identify the LRR-RLP involved in RcCDI1 recognition, we can use NbBAK1, previously shown to be an essential molecular component of the RcCDI1-triggered complex. GFP tagged-BAK1 can be overexpressed in *N. benthamiana* and immunopurified after treatment with RcCD1. The proteins associated with BAK1 will be then identified by mass spectrometry. Similar approach was followed for the identification of several proteins including the cold shock protein receptor (NbCSPR) involved in the perception of the PAMP cold shock protein (CSP) (Saur *et al.*, 2016). Another strategy involves the overexpression of RcCDI1 in transgenic *N. benthamiana* plants silenced for individual already known PRR genes. RcCDI1-triggered cell death will be compromised in the absence of the PRR required for recognition and therefore is a valuable tool to identify the LRR-RLP involved in RcDI1 recognition. Once the PRR involved in RcCDI1 recognition has been identified, future research will involve the engineering of non-host resistance to *R. commune* and other devastating fungal pathogens by the transfer of the receptor from dicots into monocot plants.

In addition, as mentioned previously, the comparison of the BgCDI1 sequence with the sequences of CDI1 from *R. commune* and its homologues triggering cell death in *N. benthamiana*, led to the discovery of 5 amino acids that might be required for CDI1 recognition. Future work should include the performance of single aminoacid substitutions to help unveiling the role of each of them for CDI1-triggered cell death.

To test the importance of Rsu3_07158 in adhesion or barley cuticle penetration, a Rsu3_07158-overexpressing *R. commune* strain can be tested for the increased pathogenicity on barley. In the same way, using the same strain, it is possible to check the protein localisation in *planta*. Besides, Rsu3_07158 protein can be purified using *P. pastoris* expression system and put in a medium containing PBSA to evaluate its role in PBSA hydrolysis, which is a similar polymer of the one found in the plant cuticle. Similar findings were obtained for the HsbA protein from *A. oryzae* (Ohtaki *et al.*, 2006). As for the Rc2 candidate gene, a future experiment involves the overexpression of this protein in *R. commune* to assess if pathogenicity is increased, a typical feature of effector proteins.

Finally, for all the proteins tested gene disruption or silencing is required to determine if any of these genes are essential for fungal pathogenicity.

In conclusion, this thesis was focused on the characterisation of *R. commune* secreted proteins. The promising results obtained in this work for the three studied proteins will be crucial in aiding our efforts to understand *R. commune*-barley interactions and, consequently, will help us achieve effective disease management strategies against *R. commune.*

References

Abramovitch RB. **2003**. Pseudomonas type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *The EMBO Journal* **22**: 60–69.

Abramovitch RB, Janjusevic R, Stebbins CE, Martin GB. **2006**. Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. *Proceedings*

of the National Academy of Sciences **103**: 2851–2856.

Adachi H, Nakano T, Miyagawa N, Ishihama N, Yoshioka M, Katou Y, Yaeno T, Shirasu K, Yoshioka H. **2015**. WRKY Transcription Factors Phosphorylated by MAPK Regulate a Plant Immune NADPH Oxidase in Nicotiana benthamiana. *The Plant Cell* **27**: 2645–2663.

Akiyama K, Kawazu K, Kobayashi A. **1995**. A novel method for chemo-enzymatic synthesis of elicitor-active chitosan oligomers and partially N-deacetylated chitin oligomers using N-acylated chitotrioses as substrates in a lysozyme-catalyzed transglycosylation reaction system. *Carbohydrate Research* **279**: 151–160.

Alavanja MCR. **2009**. Introduction: Pesticides Use and Exposure, Extensive Worldwide. *Reviews on Environmental Health* **24**: 303–309.

Albert I, Böhm H, Albert M, Feiler CE, Imkampe J, Wallmeroth N, Brancato C, Raaymakers TM, Oome S, Zhang H, *et al.* 2015. An RLP23–SOBIR1–BAK1 complex mediates NLP-triggered immunity. *Nature Plants* **1**: 15140.

Amselem J, Cuomo CA, van Kan JAL, Viaud M, Benito EP, Couloux A, Coutinho PM, de Vries RP, Dyer PS, Fillinger S, *et al.* 2011. Genomic Analysis of the Necrotrophic Fungal Pathogens Sclerotinia sclerotiorum and Botrytis cinerea (PM Richardson, Ed.). *PLoS Genetics* **7**: e1002230.

Armstrong MR, Whisson SC, Pritchard L, Bos JIB, Venter E, Avrova AO, Rehmany AP, Böhme U, Brooks K, Cherevach I, *et al.* 2005. An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 7766–71.

Aroul-Selvam R, Hubbard T, Sasidharan R. **2004**. Domain Insertions in Protein Structures. *Journal of Molecular Biology* **338**: 633–641.

Arzanlou M, Karimi K, Mirabi F. **2016**. Some evidence for skewed mating type distribution in Iranian populations of Rhynchosporium commune, the cause of barley scald disease. *Journal of Plant Protection Research* **56**.

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu W-L, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J. **2002**. MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* **415**: 977–83.

Atkinson NJ, Lilley CJ, Urwin PE. **2013**. Identification of genes involved in the response of Arabidopsis to simultaneous biotic and abiotic stresses. *Plant Physiology* **162**: 2028–2041.

Austin MJ. **2002**. Regulatory Role of SGT1 in Early R Gene-Mediated Plant Defenses. *Science* **295**: 2077–2080.

Ausubel F. **2005**. Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol* **6**: 973–979.

Avrova A, Knogge W. **2012**. Rhynchosporium commune: a persistent threat to barley cultivation. *Molecular Plant Pathology* **13**: 986–997.

Avrova AO, Venter E, Birch PR., Whisson SC. **2003**. Profiling and quantifying differential gene transcription in Phytophthora infestans prior to and during the early stages of potato infection. *Fungal Genetics and Biology* **40**: 4–14.

Azevedo C. **2002**. The RAR1 Interactor SGT1, an Essential Component of R Gene-Triggered Disease Resistance. *Science* **295**: 2073–2076.

Azevedo C, Betsuyaku S, Peart J, Takahashi A, Noël L, Sadanandom A, Casais C, Parker J, Shirasu K. **2006**. Role of SGT1 in resistance protein accumulation in plant immunity. *The EMBO Journal* **25**: 2007–2016.

Bailey BA. **1995**. Purification of a Protein from Culture Filtrates of Fusarium oxysporum that Induces Ethylene and Necrosis in Leaves of Erythroxylum coca. *Phytopathology* **85**: 1250.

Bailey BA, Dean JF, Anderson JD. **1990**. An Ethylene Biosynthesis-Inducing Endoxylanase Elicits Electrolyte Leakage and Necrosis in Nicotiana tabacum cv Xanthi Leaves. *Plant physiology* **94**: 1849–54.

Barcellos FG, Fungaro MHP, Furlaneto MC, Lejeune B, Pizzirani-Kleiner AA, Lúcio de Azevedo J. **1998**. Genetic analysis of Aspergillus nidulans unstable transformants obtained by the biolistic process. *Canadian Journal of Microbiology* **44**: 1137–1141.

Bari R, Jones JDG. **2009**. Role of plant hormones in plant defence responses. *Plant Molecular Biology* **69**: 473–488.

Bauer Z, Gómez-Gómez L, Boller T, Felix G. **2001**. Sensitivity of Different Ecotypes and Mutants of Arabidopsis thaliana toward the Bacterial Elicitor Flagellin Correlates with the Presence of Receptor-binding Sites. *Journal of Biological Chemistry* **276**: 45669–45676.

Bebber DP, Ramotowski MAT, Gurr SJ. **2013**. Crop pests and pathogens move polewards in a warming world. *Nature Climate Change* **3**: 985–988.

Benedetti M, Pontiggia D, Raggi S, Cheng Z, Scaloni F, Ferrari S, Ausubel FM, Cervone F, De Lorenzo G. **2015**. Plant immunity triggered by engineered in vivo release of oligogalacturonides, damage-associated molecular patterns. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)* **112**: 201504154.

Benschop JJ, Mohammed S, O'flaherty M, Heck AJR, Slijper M, Menke FLH. **2007**. Quantitative

Phosphoproteomics of Early Elicitor Signaling in Arabidopsis. *Molecular & Cellular Proteomics* **6.7**: 1198–1214.

Bhadauria V, Banniza S, Wei Y, Peng Y-L. **2009**. Reverse Genetics for Functional Genomics of Phytopathogenic Fungi and Oomycetes. *Comparative and Functional Genomics* **2009**: 1–11.

Bhat BS, Shahnaz E. **2014**. Effectors-Role in Host-Pathogen Interaction extracellular space , whereby they target extracellular targets and surface receptors through specialized structures like infection vesicles and haustoria that invaginate. *Journal of Agriculture and Environmental Sciences* **3**: 265–285.

Van Der Biezen EA, Jones JDG. **1998**. Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical Sciences* **23**: 454–456.

Bigeard J, Colcombet J, Hirt H. **2015**. Signaling mechanisms in pattern-triggered immunity (PTI). *Molecular Plant* **8**: 521–539.

Bjørnstad Å, Gronnerod S, Mac Key J, Tekauz A, Crossa J, Marten H. **2004**. Resistance to barley scald (Rhynchosporium secalis) in the Ethiopian donor lines 'Steudelli' and 'Jet', analyzed by partial least squares regression and interval mapping. *Hereditas* **141**: 166–179.

Bjørnstad Å, Patil V, Tekauz A, Marøy AG, Skinnes H, Jensen A, Magnus H, MacKey J. **2002**. Resistance to Scald (Rhynchosporium secalis) in Barley (Hordeum vulgare) Studied by Near-Isogenic Lines: I. Markers and Differential Isolates. *Phytopathology* **92**: 710–720.

Boevink PC, Wang X, McLellan H, He Q, Naqvi S, Armstrong MR, Zhang W, Hein I, Gilroy EM, Tian Z, *et al.* 2016. A Phytophthora infestans RXLR effector targets plant PP1c isoforms that promote late blight disease. *Nature communications* **7**: 10311.

Böhm H, Albert I, Oome S, Raaymakers TM, Van den Ackerveken G, Nürnberger T. **2014**. A Conserved Peptide Pattern from a Widespread Microbial Virulence Factor Triggers Pattern-Induced Immunity in Arabidopsis (P Birch, Ed.). *PLoS Pathogens* **10**: e1004491.

Bollen GJ, Scholten G. **1971**. Acquired resistance to benomyl and some other systemic fungicides in a strain of Botrytis cinerea in cyclamen. *Netherlands Journal of Plant Pathology* **77**: 83–90.

Boller T. **1995**. Chemoperception of Microbial Signals in Plant Cells. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**: 189–214.

Boller T, Felix G. **2009**. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual Review of Plant Biology* **60**: 379–406.

Bos JIB, Armstrong MR, Gilroy EM, Boevink PC, Hein I, Taylor RM, Zhendong T, Engelhardt S, Vetukuri RR, Harrower B, *et al.* 2010. Phytophthora infestans effector AVR3a is essential for

virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proceedings of the National Academy of Sciences* **107**: 9909–9914.

Bos JIB, Kanneganti TD, Young C, Cakir C, Huitema E, Win J, Armstrong MR, Birch PRJ, Kamoun S. **2006**. The C-terminal half of Phytophthora infestans RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in Nicotiana benthamiana. *Plant Journal* **48**: 165–176.

Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng S-H, Sheen J. **2010**. Differential innate immune signalling via Ca2+ sensor protein kinases. *Nature* **464**: 418–422.

Bouwmeester K, Han M, Blanco-Portales R, Song W, Weide R, Guo L-Y, van der Vossen EAG, Govers F. **2014**. The Arabidopsis lectin receptor kinase LecRK-I.9 enhances resistance to Phytophthora infestans in Solanaceous plants. *Plant Biotechnology Journal* **12**: 10–16.

Bozkurt TO, Schornack S, Win J, Shindo T, Ilyas M, Oliva R, Cano LM, Jones a. ME, Huitema E, van der Hoorn R a. L, *et al.* 2011. Phytophthora infestans effector AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. *Proceedings of the National Academy of Sciences* **108**: 20832–20837.

Brunner F. **2002**. Pep-13, a plant defense-inducing pathogen-associated pattern from Phytophthora transglutaminases. *The EMBO Journal* **21**: 6681–6688.

Busari MA, Kukal SS, Kaur A, Bhatt R, Dulazi AA. **2015**. Conservation tillage impacts on soil, crop and the environment. *International Soil and Water Conservation Research* **3**: 119–129.

Cai R, Lewis J, Yan S, Liu H, Clarke CR, Campanile F, Almeida NF, Studholme DJ, Lindeberg M, Schneider D, *et al.* 2011. The Plant Pathogen Pseudomonas syringae pv. tomato Is Genetically Monomorphic and under Strong Selection to Evade Tomato Immunity (DS Guttman, Ed.). *PLoS Pathogens* **7**: e1002130.

Carvalho NDSP, Arentshorst M, Jin Kwon M, Meyer V, Ram AFJ. **2010**. Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. *Applied Microbiology and Biotechnology* **87**: 1463–1473.

Catanzariti AM, Dodds PN, Ellis JG. **2007**. Avirulence proteins from haustoria-forming pathogens. *FEMS Microbiology Letters* **269**: 181–188.

Cecchini NM, Monteoliva MI, Blanco F, Holuigue L, Álvarez ME. **2009**. Features of basal and race-specific defences in photosynthetic Arabidopsis thaliana suspension cultured cells: Short communication. *Molecular Plant Pathology* **10**: 305–310.

Chakraborty BN, Kapoor M. **1990**. Transformation of filamentous fungi by electroporation. *Nucleic*

Acids Research **18**: 6737–6737.

Chakraborty BN, Patterson N a, Kapoor M. **1991**. An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi. *Canadian Journal of Microbiology* **37**: 858–863.

Chaparro-Garcia A, Schwizer S, Sklenar J, Yoshida K, Petre B, Bos JIB, Schornack S, Jones AME, Bozkurt TO, Kamoun S. **2015**. Phytophthora infestans RXLR-WY effector AVR3a associates with dynamin-related protein 2 required for endocytosis of the plant pattern recognition receptor FLS2. *PLoS ONE* **10**: 1–27.

Chaparro-Garcia A, Wilkinson RC, Gimenez-Ibanez S, Findlay K, Coffey MD, Zipfel C, Rathjen JP, Kamoun S, Schornack S. **2011**. The receptor-like kinase serk3/bak1 is required for basal resistance against the late blight pathogen Phytophthora infestans in Nicotiana benthamiana. *PLoS ONE* **6**.

Chapman S, Faulkner C, Kaiserli E, Garcia-Mata C, Savenkov EI, Roberts AG, Oparka KJ, Christie JM. **2008**. The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection. *Proceedings of the National Academy of Sciences* **105**: 20038–20043.

Chaudhari P, Ahmed B, Joly DL, Germain H. **2014**. Effector biology during biotrophic invasion of plant cells. *Virulence* **5**: 703–709.

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JDG, Felix G, Boller T. **2007**. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448**: 497–500.

Choquer M, Robin G, Le Pêcheur P, Giraud C, Levis C, Viaud M. **2008**. Ku70 or Ku80 deficiencies in the fungus Botrytis cinerea facilitate targeting of genes that are hard to knock out in a wild-type context. *FEMS Microbiology Letters* **289**: 225–232.

Clikeman JA, Khalsa GJ, Barton SL, Nickoloff JA. **2001**. Homologous recombinational repair of double-strand breaks in yeast is enhanced by MAT heterozygosity through yKU-dependent and -independent mechanisms. *Genetics* **157**: 579–589.

Conner AJ, Glare TR, Nap J-P. **2003**. The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. *The Plant Journal* **33**: 19–46.

Couch BC, Kohn LM. **2002**. A multilocus gene genealogy concordant with host preference indicates segregation of a new species , Magnaporthe oryzae , from M . grisea. *Mycological Society of America* **94**: 683–693.

Couto D, Zipfel C. **2016**. Regulation of pattern recognition receptor signalling in plants. *Nature*

Reviews Immunology **16**: 537–552.

Cuzick A, Maguire K, Hammond-Kosack KE. **2009**. Lack of the plant signalling component SGT1b enhances disease resistance to Fusarium culmorum in Arabidopsis buds and flowers. *New Phytologist* **181**: 901–912.

D.A. Jones, M.J. Dickinson, P.J. Balint-Kurti, M.S. Dixon JD. J. **1993**. Two complex resistance loci revealed in tomato by classical and RFLP mapping of the Cf-2, Cf-4, Cf-5, and Cf-9 genes for resistance to Cladosporium fulvum. *Mpmi* **6**: 348–357.

van Damme M, Bozkurt TO, Cakir C, Schornack S, Sklenar J, Jones AME, Kamoun S. **2012**. The Irish Potato Famine Pathogen Phytophthora infestans Translocates the CRN8 Kinase into Host Plant Cells. *PLoS Pathogens* **8**.

Dangl JL. **1996**. Death Don't Have No Mercy: Cell Death Programs in Plant-Microbe Interactions. *The Plant cell* **8**: 1793–1807.

Dangl JL, Jones JD. **2001**. Plant pathogens and integrated defence responses to infection. *Nature* **411**: 826–833.

Davis H, Fitt BDL. **1990**. Symptomless infection of Rhynchosporium secalis on leaves of winter barley. *Mycological Research* **94**: 557–560.

Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, *et al.* 2012. The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* **13**: 414–430.

Diamond J. **2002**. Evolution, consequences and future of plant and animal domestication. *Nature* **418**: 700–707.

Dickman MB, de Figueiredo P. **2011**. Comparative Pathobiology of Fungal Pathogens of Plants and Animals (J Heitman, Ed.). *PLoS Pathogens* **7**: e1002324.

Dickman MB, de Figueiredo P. **2013**. Death Be Not Proud-Cell Death Control in Plant Fungal Interactions. *PLoS Pathogens* **9**: 1–4.

Dinoor A, Eshed N. **1984**. The Role and Importance of Pathogens in Natural Plant Communities. *Annual Review of Phytopathology* **22**: 443–466.

Dixon GR. **2012**. Climate change – impact on crop growth and food production, and plant pathogens. *Canadian Journal of Plant Pathology* **34**: 362–379.

Dodds PN, Rathjen JP. **2010**. Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics* **11**: 539–548.

Doehlemann G, Hemetsberger C. **2013**. Apoplastic immunity and its suppression by filamentous plant pathogens. *New Phytologist* **198**: 1001–1016.

Dou D, Zhou J-M. **2012**. Phytopathogen Effectors Subverting Host Immunity: Different Foes, Similar Battleground. *Cell Host & Microbe* **12**: 484–495.

Du Y, Mpina MH, Birch PRJ, Bouwmeester K, Govers F. **2015**. Phytophthora infestans RXLR effector AVR1 interacts with exocyst component Sec5 to manipulate plant immunity. *Plant Physiology* **169**: 1975–1990.

Dunning FM, Sun W, Jansen KL, Helft L, Bent AF. **2007**. Identification and mutational analysis of Arabidopsis FLS2 leucine-rich repeat domain residues that contribute to flagellin perception. *The Plant cell* **19**: 3297–3313.

Durrant WE. **2000**. cDNA-AFLP Reveals a Striking Overlap in Race-Specific Resistance and Wound Response Gene Expression Profiles. *The Plant cell* **12**: 963–977.

Dyck PL, Schaller CW. **1961**. Association of two genes for scald resistance with a specific barley chromosome. *Canadian Journal of Genetics and Cytology* **3**: 165–169.

Espinosa A, Guo M, Tam VC, Fu ZQ, Alfano JR. **2003**. The Pseudomonas syringae type III-secreted protein HopPtoD2 possesses protein tyrosine phosphatase activity and suppresses programmed cell death in plants. *Molecular Microbiology* **49**: 377–387.

Eulgem T, Rushton PJ, Robatsek S, Somssich E. **2000**. The WRKY superfamily of plant transcription factors. *Trends in Plant Science* **5**: 199–206.

Feldman HJ. **2012**. Identifying structural domains of proteins using clustering. *BMC Bioinf.* **13**: 286.

Felix G, Boller T. **2003**. Molecular sensing of bacteria in plants: The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. *Journal of Biological Chemistry* **278**: 6201–6208.

Felix G, Duran JD, Volko S, Boller T. **1999**. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant Journal* **18**: 265–276.

Fellbrich G, Romanski A, Varet A, Blume B, Brunner F, Engelhardt S, Felix G, Kemmerling B, Krzymowska M, Nürnberger T. **2002**. NPP1, a Phytophthora -associated trigger of plant defense in parsley and Arabidopsis. *The Plant Journal* **32**: 375–390.

Fernandez Acero FJ, Carbú M, El-Akhal MR, Garrido C, González-Rodríguez VE, Cantoral JM. **2011**. Development of proteomics-based fungicides: New strategies for environmentally friendly control of fungal plant diseases. *International Journal of Molecular Sciences* **12**: 795–816.

Fesel PH, Zuccaro A. **2016**. β-glucan: Crucial component of the fungal cell wall and elusive MAMP in plants. *Fungal Genetics and Biology* **90**: 53–60.

Fiegen M, Knogge W. **2002**. Amino acid alterations in isoforms of the effector protein NIP1 from Rhynchosporium secalis have similar effects on its avirulence- and virulence-associated activities on barley. *Physiological and Molecular Plant Pathology* **61**: 299–302.

Fitt BDL, Mccartney HA, Creighton NF, Lacey ME, Walklate PJ. **1988**. Dispersal of Rhynchosporium secalis conidia from infected barley leaves or straw by simulated rain. *Annuals of Applied Biology* **112**: 49–59.

Flor H. **1955**. Host-parasite interactions in flax rust: its genetics and other implications. *Phytopathology* **45**: 680–685.

Flor H. **1971**. Current Status of the Gene-For-Gene Concept. *Annual Review of Phytopathology* **9**: 275–296.

Foster SJ, Fitt BDL. **2003**. Isolation and characterisation of the mating-type (MAT) locus from Rhynchosporium secalis. *Current Genetics* **44**: 277–286.

Fradin EF, Abd-El-Haliem A, Masini L, van den Berg GCM, Joosten MH a J, Thomma BPHJ. **2011**. Interfamily Transfer of Tomato Ve1 Mediates Verticillium Resistance in Arabidopsis. *Plant Physiology* **156**: 2255–2265.

Gachon C, Saindrenan P. **2004**. Real-time PCR monitoring of fungal development in Arabidopsis thaliana infected by Alternaria brassicicola and Botrytis cinerea. *Plant Physiology and Biochemistry* **42**: 367–371.

Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, FitzHugh W, Ma L-J, Smirnov S, Purcell S, *et al.* 2003. The genome sequence of the filamentous fungus Neurospora crassa. *Nature* **422**: 859–868.

Galan J, Wolf-Watz H. **2006**. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444**: 567–573.

Gao M, Wang X, Wang D, Xu F, Ding X, Zhang Z, Bi D, Cheng YT, Chen S, Li X, *et al.* 2009. Regulation of Cell Death and Innate Immunity by Two Receptor-like Kinases in Arabidopsis. *Cell Host & Microbe* **6**: 34–44.

Gijzen M, Nürnberger T. **2006**. Nep1-like proteins from plant pathogens: Recruitment and diversification of the NPP1 domain across taxa. *Phytochemistry* **67**: 1800–1807.

Gilroy EM, Taylor RM, Hein I, Boevink P, Sadanandom A, Birch PRJ. **2011**. CMPG1-dependent cell death follows perception of diverse pathogen elicitors at the host plasma membrane and is

suppressed by Phytophthora infestans RXLR effector AVR3a. *New Phytologist* **190**: 653–666.

Gimenez-Ibanez S, Hann DR, Ntoukakis V, Petutschnig E, Lipka V, Rathjen JP. **2009**. AvrPtoB Targets the LysM Receptor Kinase CERK1 to Promote Bacterial Virulence on Plants. *Current Biology* **19**: 423–429.

Glazebrook J. **2005**. Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. *Annual Review of Phytopathology* **43**: 205–227.

Göhre V, Robatzek S. **2008**. Breaking the Barriers: Microbial Effector Molecules Subvert Plant Immunity. *Annual Review of Phytopathology* **46**: 189–215.

Göhre V, Spallek T, Häweker H, Mersmann S, Mentzel T, Boller T, de Torres M, Mansfield JW, Robatzek S. **2008**. Plant Pattern-Recognition Receptor FLS2 Is Directed for Degradation by the Bacterial Ubiquitin Ligase AvrPtoB. *Current Biology* **18**: 1824–1832.

Goldman GH, Van Montagu M, Herrera-Estrella A. **1990**. Transformation of Trichoderma harzianum by high-voltage electric pulse. *Current Genetics* **17**: 169–174.

Gómez-Gómez L, Boller T. **2000**. FLS2. *Molecular Cell* **5**: 1003–1011.

Gómez-Gómez L, Boller T. **2002**. Flagellin perception: A paradigm for innate immunity. *Trends in Plant Science* **7**: 251–256.

Gómez-Gómez L, Felix G, Boller T. **1999**. A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. *Plant Journal* **18**: 277–284.

Gonzalez-Lamothe R. **2006**. The U-Box Protein CMPG1 Is Required for Efficient Activation of Defense Mechanisms Triggered by Multiple Resistance Genes in Tobacco and Tomato. *The Plant cell* **18**: 1067–1083.

Govrin EM, Levine A. **2000**. The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. *Current Biology* **10**: 751–757.

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J. **2000**. The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *The Plant Journal* **23**: 441–450.

Greeff C, Roux M, Mundy J, Petersen M. **2012**. Receptor-like kinase complexes in plant innate immunity. *Frontiers in Plant Science* **3**: 1–7.

Grenville-Briggs LJ, Anderson VL, Fugelstad J, Avrova AO, Bouzenzana J, Williams A, Wawra S, Whisson SC, Birch PRJ, Bulone V, *et al.* 2008. Cellulose Synthesis in Phytophthora infestans Is Required for Normal Appressorium Formation and Successful Infection of Potato. *the plant cell* **20**:

720–738.

De Groot PWJ, De Boer AD, Cunningham J, Dekker HL, De Jong L, Hellingwerf KJ, De Koster C, Klis FM. **2004**. Proteomic Analysis of Candida albicans cell walls revelas covalently bound carbohydrate-active enzymes and adhesins. *Euka* **3**: 955–965.

de Groot MJA, Bundock P, Hooykaas PJ., Beijersbergen AGM. **1998**. Agrobacterium tumefaciens-mediated transformation of filamentous fungi. *Nature Biotechnology* **16**: 839–842.

Gu Y-Q, Wildermuth MC, Chakravarthy S, Loh Y-T, Yang C, He X, Han Y, Martin GB. **2002**. Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in Arabidopsis. *The Plant cell* **14**: 817–31.

Guo M, Tian F, Wamboldt Y, Alfano JR. **2009**. The Majority of the Type III Effector Inventory of Pseudomonas syringae pv. tomato DC3000 Can Suppress Plant Immunity. *Molecular Plant-Microbe Interactions* **22**: 1069–1080.

Gürlebeck D, Szurek B, Bonas U. **2005**. Dimerization of the bacterial effector protein AvrBs3 in the plant cell cytoplasm prior to nuclear import. *The Plant Journal* **42**: 175–187.

Gust AA, Biswas R, Lenz HD, Rauhut T, Ranf S, Kemmerling B, Gotz F, Glawischnig E, Lee J, Felix G, *et al.* 2007. Bacteria-derived Peptidoglycans Constitute Pathogen-associated Molecular Patterns Triggering Innate Immunity in Arabidopsis. *Journal of Biological Chemistry* **282**: 32338–32348.

Haas BJ, Kamoun S, Zody MC, Jiang RHY, Handsaker RE, Cano LM, Grabherr M, Kodira CD, Raffaele S, Torto-Alalibo T, *et al.* 2009. Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. *Nature* **461**: 393–8.

Hahn M, Jüngling S, Knogge W. **1993**. Cultivar-specific elicitation of barley defense reactions by the phytotoxic peptide NIP1 from Rhynchosporium secalis. *Molecular plant-microbe interactions : MPMI* **6**: 745–54.

Halim VA, Hunger A, Macioszek V, Landgraf P, Nürnberger T, Scheel D, Rosahl S. **2004**. The oligopeptide elicitor Pep-13 induces salicylic acid-dependent and -independent defense reactions in potato. *Physiological and Molecular Plant Pathology* **64**: 311–318.

Hanin M, Paszkowski J. **2003**. Plant genome modification by homologous recombination. *Current Opinion in Plant Biology* **6**: 157–162.

Hann DR, Rathjen JP. **2007**. Early events in the pathogenicity of Pseudomonas syringae on Nicotiana benthamiana. *The Plant Journal* **49**: 607–618.

Hao G, Pitino M, Ding F, Lin H, Stover E, Duan Y. **2014**. Induction of innate immune responses by

flagellin from the intracellular bacterium, 'CandidatusLiberibacter solanacearum'. *BMC Plant Biology* **14**: 211.

Hausbeck MK, Lamour KH. **2004**. Research Progress and Management Challenges Phytophthora capsici on Vegetable Crops : *Plant Disease* **88**: 1292–1303.

Haverkort AJ, Boonekamp PM, Hutten R, Jacobsen E, Lotz LAP, Kessel GJT, Visser RGF, Van Der Vossen EAG. **2008**. Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. *Potato Research* **51**: 47–57.

Hayafune M, Berisio R, Marchetti R, Silipo A, Kayama M, Desaki Y, Arima S, Squeglia F, Ruggiero A, Tokuyasu K, *et al.* 2014. Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. *Proceedings of the National Academy of Sciences* **111**: E404–E413.

Hecht V, Vielle-Calzada JP, Hartog M V, Schmidt ED, Boutilier K, Grossniklaus U, de Vries SC. **2001**. The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant physiology* **127**: 803–16.

Heese A, Hann DR, Gimenez-Ibanez S, Jones AME, He K, Li J, Schroeder JI, Peck SC, Rathjen JP. **2007**. The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 12217–12222.

Hind SR, Strickler SR, Boyle PC, Dunham DM, Bao Z, O'Doherty IM, Baccile JA, Hoki JS, Viox EG, Clarke CR, *et al.* 2016. Tomato receptor FLAGELLIN-SENSING 3 binds flgII-28 and activates the plant immune system. *Nature Plants* **2**: 16128.

Holton N, Nekrasov V, Ronald PC, Zipfel C. **2015**. The Phylogenetically-Related Pattern Recognition Receptors EFR and XA21 Recruit Similar Immune Signaling Components in Monocots and Dicots (W Ma, Ed.). *PLOS Pathogens* **11**: e1004602.

Van der Hoorn RAL, Kamoun S. **2008**. From Guard to Decoy: A New Model for Perception of Plant Pathogen Effectors. *The Plant cell* **20**: 2009–2017.

Van der Hoorn RAL, Laurent F, Roth R, De Wit PJGM. **2000**. Agroinfiltration Is a Versatile Tool That Facilitates Comparative Analyses of Avr 9/ Cf-9 -Induced and Avr 4/ Cf-4 -Induced Necrosis. *Molecular Plant-Microbe Interactions* **13**: 439–446.

Van den Hooven HW, Van den Burg HA, Vossen P, Boeren S, de Wit PJGM, Vervoort J. **2001**. Disulfide Bond Structure of the AVR9 Elicitor of the Fungal Tomato Pathogen Cladosporium fulvum : Evidence for a Cystine Knot †. *Biochemistry* **40**: 3458–3466.

Hua S, Qiu M, Chan E, Zhu L, Luo Y. **1997**. Minimum Length of Sequence Homology Required forin VivoCloning by Homologous Recombination in Yeast. *Plasmid* **38**: 91–96.

Hwang SF, Ahmed HU, Zhou Q, Turnbull GD, Strelkov SE, Gossen BD, Peng G. **2015**. Effect of host and non-host crops on Plasmodiophora brassicae resting spore concentrations and clubroot of canola. *Plant Pathology* **64**: 1198–1206.

Imam J, Singh PK, Shukla P. **2016**. Plant Microbe Interactions in Post Genomic Era: Perspectives and Applications. *Frontiers in Microbiology* **7**: 1–15.

Ishihama N, Yamada R, Yoshioka M, Katou S, Yoshioka H. **2011**. Phosphorylation of the Nicotiana benthamiana WRKY8 Transcription Factor by MAPK Functions in the Defense Response. *The Plant Cell* **23**: 1153–1170.

Jackson L, Webster R. **1976**. The dynamics of a controlled population of Rhynchosporium secalis, changes in race composition and frequencies. *Phytopathology* **66**: 726–728.

Jamir Y, Guo M, Oh H-S, Petnicki-Ocwieja T, Chen S, Tang X, Dickman MB, Collmer A, R. Alfano J. **2004**. Identification of Pseudomonas syringae type III effectors that can suppress programmed cell death in plants and yeast. *The Plant Journal* **37**: 554–565.

Jaskiewicz M, Conrath U, Peterhänsel C. **2010**. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *{EMBO} reports* **12**: 50–55.

Johal GS, Briggs SP. **1992**. Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science* **258**: 985–987.

Jones P, Ayres PG. **1974**. Rhynchosporium leaf blotch of barley studied during the subcuticular phase by electron microscopy. *Physiological Plant Pathology* **4**: 229–233.

Jones DA, Dickinson MJ, Balint-Kurti PJ, Dixon MS, Jones JDG. 1993. Two complex resistance loci revealed in tomato by classical and RFLP mapping of the *Cf-2*,*Cf-4*,*Cf-5* and *Cf-9* genes for resistance to *Cladosporium fulvum*. *Molecular Plant-Microbe Interactions* **6**:348-357.

Jones J, Dangl J. **2006**. The plant immune system. *Nature* **444**: 323–329.

De Jonge R, Peter van Esse H, Kombrink A, Shinya T, Desaki Y, Bours R, van der Krol S, Shibuya N, Joosten MH a J, Thomma BPHJ. **2010**. Conserved Fungal LysM Effector Ecp6 Prevents Chitin-Triggered Immunity in Plants. *Science* **329**: 953–955.

De Jonge R, Peter van Esse H, Maruthachalam K, Bolton MD, Santhanam P, Saber MK, Zhang Z, Usami T, Lievens B, Subbarao K V, *et al.* 2012. Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proceedings of the National Academy of Sciences* **109**: 5110–5115.

Joosten M. **1997**. The Biotrophic Fungus Cladosporium fulvum Circumvents Cf-4[mdash]Mediated Resistance by Producing Unstable AVR4 Elicitors. *The Plant cell* **9**: 367–379.

Joosten MHAJ, Cozijnsen TJ, De Wit PJGM. **1994**. Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. *Nature* **367**: 384–386.

Joska TM, Mashruwala A, Boyd JM, Belden WJ. **2014**. A universal cloning method based on yeast homologous recombination that is simple, efficient, and versatile. *Journal of Microbiological Methods* **100**: 46–51.

Journot-Catalino N, Somssich IE, Roby D, Kroj T. **2006**. The Transcription Factors WRKY11 and WRKY17 Act as Negative Regulators of Basal Resistance in Arabidopsis thaliana. *The Plant cell* **18**: 3289–3302.

Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K, Minami E, Shibuya N. **2006**. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 11086–11091.

Kamoun S. **1998**. Resistance of Nicotiana benthamiana to Phytophthora infestans Is Mediated by the Recognition of the Elicitor Protein INF1. *The Plant cell* **10**: 1413–1426.

Kamoun S. **2006**. A Catalogue of the Effector Secretome of Plant Pathogenic Oomycetes. *Annual Review of Phytopathology* **44**: 41–60.

Kamoun S, Lindqvist H, Govers F. **1997**. A novel class of elicitin-like genes from Phytophthora infestans. *Molecular plant-microbe interactions : MPMI* **10**: 1028–1030.

Kang L, Tang X, Mysore KS. **2004**. Pseudomonas Type III Effector AvrPto Suppresses the Programmed Cell Death Induced by Two Nonhost Pathogens in Nicotiana benthamiana and Tomato. *Molecular Plant-Microbe Interactions* **17**: 1328–1336.

Kari AG, Griffiths E. **1993**. Components of partial resistance of barley to Rhynchosporium secalis: use of seedling tests to predict field resistance. *Annals of Applied Biology* **123**: 545–561.

Karimi M, Inzé D, Depicker A. **2002**. GATEWAY^{TM} vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science* **7**: 193–195.

Karimi M, De Meyer B, Hilson P. **2005**. Modular cloning in plant cells. *Trends in Plant Science* **10**: 103–105.

Keen NT. **1990**. Gene-for-Gene Complementarity in Plant -Pathogen Interactions N. *Annual review of genetics*: 447–463.

Kettles GJ, Bayon C, Canning G, Rudd JJ, Kanyuka K. **2017**. Apoplastic recognition of multiple candidate effectors from the wheat pathogen Zymoseptoria tritici in the nonhost plant Nicotiana benthamiana. *New Phytologist* **213**: 338–350.

Khatib M, Lafitte C, Esquerre-Tugaye M-T, Bottin A, Rickauer M. **2004**. The CBEL elicitor of Phytophthora parasitica var. nicotianae activates defence in Arabidopsis thaliana via three different signalling pathways. *New Phytologist* **162**: 501–510.

Kim K-T, Jeon J, Choi J, Cheong K, Song H, Choi G, Kang S, Lee Y-H. **2016**. Kingdom-Wide Analysis of Fungal Small Secreted Proteins (SSPs) Reveals their Potential Role in Host Association. *Frontiers in Plant Science* **7**: 186.

Kim S, Park J, Park S-Y, Mitchell T, Lee Y-H. **2010**. Identification and analysis of in planta expressed genes of Magnaporthe oryzae. *BMC Genomics* **11**: 104.

King SRF, McLellan H, Boevink PC, Armstrong MR, Bukharova T, Sukarta O, Win J, Kamoun S, Birch PRJ, Banfield MJ. **2014**. Phytophthora infestans RXLR Effector PexRD2 Interacts with Host MAPKKK to Suppress Plant Immune Signaling. *The Plant Cell* **26**: 1345–1359.

King KM, West JS, Brunner PC, Dyer PS, Fitt BDL. **2013**. Evolutionary Relationships between Rhynchosporium lolii sp. nov. and Other Rhynchosporium Species on Grasses (M Vos, Ed.). *PLoS ONE* **8**: e72536.

Kirsten S, Navarro-Quezada A, Penselin D, Wenzel C, Matern A, Leitner A, Baum T, Seiffert U, Knogge W. **2012**. Necrosis-Inducing Proteins of Rhynchosporium commune , Effectors in Quantitative Disease Resistance. *Molecular Plant-Microbe Interactions* **25**: 1314–1325.

Kirsten S, Siersleben S, Knogge W. **2011**. A GFP-based assay to quantify the impact of effectors on the ex planta development of the slowly growing barley pathogen Rhynchosporium commune. *Mycologia* **103**: 1019–1027.

Klemptner RL, Sherwood JS, Tugizimana F, Dubery IA, Piater LA. **2014**. Ergosterol, an orphan fungal microbe-associated molecular pattern (MAMP). *Molecular Plant Pathology* **15**: 747–761.

Koch E, Slusarenko A. **1990**. Arabidopsis Is Susceptible to Infection by a Downy Mildew Fungus. *The Plant cell* **2**: 437–445.

Koeck M, Hardham AR, Dodds PN. **2011**. The role of effectors of biotrophic and hemibiotrophic fungi in infection. *Cellular Microbiology* **13**: 1849–1857.

Koh CMJ, Liu Y, Moehninsi, Du M, Ji L. **2014**. Molecular characterization of KU70 and KU80 homologues and exploitation of a KU70-deficient mutant for improving gene deletion frequency in Rhodosporidium toruloides. *BMC microbiology* **14**: 50.

Kohler AC, Chen L-H, Hurlburt N, Salvucci A, Schwessinger B, Fisher AJ, Stergiopoulosa I. **2016**. Chitin is a key component of fungal cell walls and a potent inducer of innate immune responses. Consequently, fungi may secrete chitin binding lectins, such as the Cf-Avr4 effector protein from the tomato pathogen Cladosporium fulvum, to shield chitin fro. *The Plant Cell* **28**: 1–21.

Kooistra R, Hooykaas PJJ, Steensma HY. **2004**. Efficient gene targeting in Kluyveromyces lactis. : 781–792.

Koukaki M, Giannoutsou E, Karagouni A, Diallinas G. **2003**. A novel improved method for Aspergillus nidulans transformation. *Journal of Microbiological Methods* **55**: 687–695.

Kubigsteltig I, Laudert D, Weiler EW. **1999**. Structure and regulation of the Arabidopsis thaliana allene oxide synthase gene. *Planta* **208**: 463–471.

Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G. **2004**. The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *The Plant cell* **16**: 3496–507.

Lacombe S, Rougon-Cardoso A, Sherwood E, Peeters N, Dahlbeck D, van Esse HP, Smoker M, Rallapalli G, Thomma BPHJ, Staskawicz B, *et al.* 2010. Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. *Nature Biotechnology* **28**: 365–369.

Laluk K, Mengiste T. **2010**. Necrotroph Attacks on Plants: Wanton Destruction or Covert Extortion? *The Arabidopsis Book* **8**: e0136.

Lee W-S, Hammond-Kosack KE, Kanyuka K. **2012**. Barley Stripe Mosaic Virus-Mediated Tools for Investigating Gene Function in Cereal Plants and Their Pathogens: Virus-Induced Gene Silencing, Host-Mediated Gene Silencing, and Virus-Mediated Overexpression of Heterologous Protein. *Plant Phisiology* **160**: 582–590.

Lee SW, Han SW, Bartley LE, Ronald PC. **2006**. Unique characteristics of Xanthomonas oryzae pv. oryzae AvrXa21 and implications for plant innate immunity. *Proc Natl Acad Sci U S A* **103**: 18395–18400.

Lee W-S, Rudd JJ, Kanyuka K. **2015**. Virus induced gene silencing (VIGS) for functional analysis of wheat genes involved in Zymoseptoria tritici susceptibility and resistance. *Fungal Genetics and Biology* **79**: 84–88.

Lehnackers H, Knogge W. **1990**. Cytological studies on the infection of barley cultivars with known resistance genotypes by Rhynchosporium secalis. *Canadian Journal of Botany* **68**: 1953–1961.

Li J, Wen J, Lease KA, Doke JT, Tax FE, Walker JC. **2002**. BAK1, an Arabidopsis LRR Receptor-like Protein Kinase, Interacts with BRI1 and Modulates Brassinosteroid Signaling. *Cell* **110**: 213–222.

Li Q, Zhang M, Shen D, Liu T, Chen Y, Zhou JM, Dou D. **2016**. A Phytophthora sojae effector

PsCRN63 forms homo-/hetero-dimers to suppress plant immunity via an inverted association manner. *Sci Rep* **6**: 26951.

Lida Y, Van 't Hof P, Beenen H, Mesarich C, Kubota M, Stergiopoulos I, Mehrabi R, Notsu A, Fujiwara K, Bahkali A, *et al.* 2015. Novel mutations detected in avirulence genes overcoming tomato Cf resistance genes in isolates of a Japanese population of Cladosporium fulvum. *PLoS ONE* **10**: 1–18.

Liebrand TWH, van den Berg GCM, Zhang Z, Smit P, Cordewener JHG, America AHP, Sklenar J, Jones AME, Tameling WIL, Robatzek S, *et al.* 2013. Receptor-like kinase SOBIR1/EVR interacts with receptor-like proteins in plant immunity against fungal infection. *Proceedings of the National Academy of Sciences* **110**: 10010–10015.

Liebrand TWH, van den Burg HA, Joosten MHAJ. **2014**. Two for all: receptor-associated kinases SOBIR1 and BAK1. *Trends in Plant Science* **19**: 123–132.

Lievens B, Houterman PM, Rep M. **2009**. Effector gene screening allows unambiguous identification of Fusarium oxysporum f. sp. lycopersici â€*ƒ*races and discrimination from other formae speciales. *FEMS Microbiology Letters* **300**: 201–215.

Linde CC, Zala M, Ceccarelli S, McDonald BA. **2003**. Further evidence for sexual reproduction in Rhynchosporium secalis based on distribution and frequency of mating-type alleles. *Fungal Genetics and Biology* **40**: 115–125.

Liu R, Chen L, Jiang Y, Zhou Z, Zou G. **2015**. Efficient genome editing in filamentous fungus Trichoderma reesei using the CRISPR/Cas9 system. *Cell Discovery* **1**: 15007.

Liu J, Liu X, Dai L, Wang G. **2007**. Recent progress in elucidating the structure, function and evolution of disease resistance genes in plants. *Journal of genetics and genomics = Yi chuan xue bao* **34**: 765–776.

Liu T, Ye W, Ru Y, Yang X, Gu B, Tao K, Lu S, Dong S, Zheng X, Shan W, *et al.* 2011. Two host cytoplasmic effectors are required for pathogenesis of Phytophthora sojae by suppression of host defenses. *Plant Physiol* **155**: 490–501.

Lorito M, Hayes CK, Di Pietro A, Harman GE. **1993**. Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. *Curr Genet* **24**: 349–35.

Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J. **2011**. Callose deposition: a multifaceted plant defense response. *Molecular Plant-Microbe Interactions* **24**: 183–193.

Ma Z, Song T, Zhu L, Ye W, Wang Y, Shao Y, Dong S, Zhang Z, Dou D, Zheng X, *et al.* 2015. A Phytophthora sojae Glycoside Hydrolase 12 Protein Is a Major Virulence Factor during Soybean

Infection and Is Recognized as a PAMP. *The Plant Cell* **27**: 2057–2072.

Ma X, Xu G, He P, Shan L. **2016**. SERKing Coreceptors for Receptors. *Trends in Plant Science* **21**: 1017–1033.

Mackey D, Holt BF, Wiig A, Dangl JL. **2002**. RIN4 Interacts with Pseudomonas syringae Type III Effector Molecules and Is Required for RPM1-Mediated Resistance in Arabidopsis. *Cell* **108**: 743–754.

Martin GB, Bogdanove AJ, Sessa G. **2003**. UNDERSTANDING THE FUNCTIONS OF PLANT DISEASE RESISTANCE PROTEINS. *Annual Review of Plant Biology* **54**: 23–61.

Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD. **1993**. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science (New York, N.Y.)* **262**: 1432–1436.

Matsu-ura T, Baek M, Kwon J, Hong C. **2015**. Efficient gene editing in Neurospora crassa with CRISPR technology. *Fungal Biology and Biotechnology* **2**: 4.

McDonald BA. **2015**. How can research on pathogen population biology suggest disease management strategies? The example of barley scald (Rhynchosporium commune). *Plant Pathology* **64**: 1005–1013.

McLellan H, Boevink PC, Armstrong MR, Pritchard L, Gomez S, Morales J, Whisson SC, Beynon JL, Birch PRJ. **2013**. An RxLR Effector from Phytophthora infestans Prevents Re-localisation of Two Plant NAC Transcription Factors from the Endoplasmic Reticulum to the Nucleus. *PLoS Pathogens* **9**.

Medzhitov R, Janeway CAJ. **1997**. Innate immunity: Minireview the virtues of a nonclonal system of recognition. *Cell* **91**: 295–298.

Mei C, Qi M, Sheng G, Yang Y. **2006**. of a Rice Allene Oxide Synthase Gene Increases the Endogenous Jasmonic Acid Level , PR Gene Expression , and Host Resistance to Fungal Infection. **19**: 1127–1137.

Melech-Bonfil S, Sessa G. **2010**. Tomato MAPKKKε is a positive regulator of cell-death signaling networks associated with plant immunity. *Plant Journal* **64**: 379–391.

Mendes BMJ, Cardoso SC, Boscariol-Camargo RL, Cruz RB, MourÃ£o Filho FAA, Bergamin Filho A. **2010**. Reduction in susceptibility to Xanthomonas axonopodis pv. citri in transgenic Citrus sinensis expressing the rice Xa21 gene. *Plant Pathology* **59**: 68–75.

Mendgen K, Hahn M. **2002**. Plant infection and the establishment of fungal biotrophy. *Trends in Plant Science* **7**: 352–356.

Mishina TE, Zeier J. **2007**. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *Plant Journal* **50**: 500–513.

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N. **2007**. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences* **104**: 19613–19618.

Mueller MJ. **1997**. Enzymes involved in jasmonic acid biosynthesis. *Physiologia Plantarum* **100**: 653–663.

Münch S, Lingner U, Floss DS, Ludwig N, Sauer N, Deising HB. **2008**. The hemibiotrophic lifestyle of Colletotrichum species. *Journal of Plant Physiology* **165**: 41–51.

Muthamilarasan M, Prasad M. **2013**. Plant innate immunity: An updated insight into defense mechanism. *Journal of Biosciences* **38**: 433–449.

Mysore KS, Ryu C-M. **2004**. Nonhost resistance: how much do we know? *Trends in Plant Science* **9**: 97–104.

Naito K, Ishiga Y, Toyoda K, Shiraishi T, Ichinose Y. **2007**. N-terminal domain including conserved flg22 is required for flagellin-induced hypersensitive cell death in Arabidopsis thaliana. *Journal of General Plant Pathology* **73**: 281–285.

Naito K, Taguchi F, Suzuki T, Inagaki Y, Toyoda K, Shiraishi T, Ichinose Y. **2008**. Amino Acid Sequence of Bacterial Microbe-Associated Molecular Pattern flg22 Is Required for Virulence. *Molecular Plant-Microbe Interactions* **21**: 1165–1174.

Nakagami H, Sugiyama N, Mochida K, Daudi A, Yoshida Y, Toyoda T, Tomita M, Ishihama Y, Shirasu K. **2010**. Large-Scale Comparative Phosphoproteomics Identifies Conserved Phosphorylation Sites in Plants. *Plant Physiology* **153**: 1161–1174.

Nayak T. **2006**. A Versatile and Efficient Gene-Targeting System for Aspergillus nidulans. *Genetics* **172**: 1557–1566.

Nemri A, Saunders DGO, Anderson C, Upadhyaya NM, Win J, Lawrence GJ, Jones DA, Kamoun S, Ellis JG, Dodds PN. **2014**. The genome sequence and effector complement of the flax rust pathogen Melampsora lini. *Frontiers in plant science* **5**: 98.

Newton AC. **1989**. Somatic recombination in Rhynchosporium secalis. *Plant Pathology* **38**: 71–74.

Newton A, Searle J, Guy DC, Hackett CA, Cooke D. **2001**. Variability in pathotype , aggressiveness , RAPD profile , and rDNA ITS1 sequences of UK isolates of [i]Rhynchosporium secalis[/i]. : 446–458.

Nicaise V, Roux M, Zipfel C. **2009**. Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiology* **150**: 1638–1647.

Ninomiya Y, Suzuki K, Ishii C, Inoue H. **2004**. Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining. *Proceedings of the National Academy of Sciences* **101**: 12248–12253.

Noar RD, Daub ME. **2016**. Bioinformatics prediction of polyketide synthase gene clusters from Mycosphaerella fijiensis. *PLoS ONE* **11**: 1–31.

Nürnberger T, Brunner F. **2002**. Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. *Current Opinion in Plant Biology* **5**: 318–324.

Nurnberger T, Brunner F, Kemmerling B, Piater L. **2004**. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews* **198**: 249–266.

Oerke E-C. **2006**. Crop losses to pests. *The Journal of Agricultural Science* **144**: 31.

Oh CS, Martin GB. **2011**. Tomato 14-3-3 protein TFT7 interacts with a MAP kinase kinase to regulate immunity-associated programmed cell death mediated by diverse disease resistance proteins. *Journal of Biological Chemistry* **286**: 14129–14136.

Ohtaki S, Maeda H, Takahashi T, Hasegawa F, Gomi K, Abe K, Yamagata Y, Nakajima T. **2006**. Novel Hydrophobic Surface Binding Protein , HsbA , Produced by Aspergillus oryzae Novel Hydrophobic Surface Binding Protein , HsbA , Produced by Aspergillus oryzae. *Applied and environmental microbiology* **72**: 2407–2413.

El Oirdi M, Bouarab K. **2007**. Plant signalling components EDS1 and SGT1 enhance disease caused by the necrotrophic pathogen Botrytis cinerea. *New Phytologist* **175**: 131–139.

Oldenburg K. **1997**. Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Research* **25**: 451–452.

Oliver RP, Ipcho SVS. **2004**. Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens. *Molecular Plant Pathology* **5**: 347–352.

Oome S, Van den Ackerveken G. **2014**. Comparative and Functional Analysis of the Widely Occurring Family of Nep1-Like Proteins. *Molecular Plant-Microbe Interactions* **27**: 1081–1094.

Oome S, Raaymakers TM, Cabral A, Samwel S, Böhm H, Albert I, Nürnberger T, Van den Ackerveken G. **2014**. Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in Arabidopsis. *Proceedings of the National Academy of Sciences* **111**: 16955–16960.

Orton ES, Brown JKM. **2016**. Reduction of Growth and Reproduction of the Biotrophic Fungus Blumeria graminis in the Presence of a Necrotrophic Pathogen. *Frontiers in Plant Science* **7**: 1–12.

Ottmann C, Luberacki B, Kufner I, Koch W, Brunner F, Weyand M, Mattinen L, Pirhonen M, Anderluh G, Seitz HU, *et al.* 2009. A common toxin fold mediates microbial attack and plant defense. *Proceedings of the National Academy of Sciences* **106**: 10359–10364.

Paietta J V, Marzluf G a. **1985**. Gene disruption by transformation in Neurospora crassa. *Molecular and cellular biology* **5**: 1554–9.

Parra G, Ristaino JB. **2001**. Resistance to Mefenoxam and Metalaxyl Among Field Isolates of Phytophthora capsici Causing Phytophthora Blight of Bell Pepper. *Plant Disease* **85**: 1069–1075.

Pazzagli L, Pantera B, Carresi L, Zoppi C, Pertinhez TA, Spisni A, Tegli S, Scala A, Cappugi G. **2006**. Cerato-Platanin, the First Member of a New Fungal Protein Family: Cloning, Expression, and Characterization. *Cell Biochemistry and Biophysics* **44**: 512–521.

Peart JR, Lu R, Sadanandom A, Malcuit I, Moffett P, Brice DC, Schauser L, Jaggard D a W, Xiao S, Coleman MJ, *et al.* 2002. Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 10865–10869.

Pedley KF, Martin GB. **2003**. Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. *Annual Review of Phytopathology* **41**: 215–243.

Pemberton CL, Salmond GPC. **2004**. The Nep1-like proteins - A growing family of microbial elicitors of plant necrosis. *Molecular Plant Pathology* **5**: 353–359.

Penselin D, Münsterkötter M, Kirsten S, Felder M, Taudien S, Platzer M, Ashelford K, Paskiewicz KH, Harrison RJ, Hughes DJ, *et al.* 2016. Comparative genomics to explore phylogenetic relationship, cryptic sexual potential and host specificity of Rhynchosporium species on grasses. *BMC Genomics* **17**: 953.

Perfect SE, Green JR. **2001**. Infection structures of biotrophic and hemibiotrophic fungal plant pathogens. *Molecular Plant Pathology* **2**: 101–108.

Petre B, Kamoun S. **2014**. How Do Filamentous Pathogens Deliver Effector Proteins into Plant Cells? (JM McDowell, Ed.). *PLoS Biology* **12**: e1001801.

Pettolino F, Sasaki I, Turbic A, Wilson SM, Bacic A, Hrmova M, Fincher GB. **2009**. Hyphal cell walls from the plant pathogen Rhynchosporium secalis contain (1,3/1,6)-??-D-glucans, galacto- and rhamnomannans, (1,3;1,4)-??-D- glucans and chitin. *FEBS Journal* **276**: 4122–4133.

Ploetz RC. **2000**. Panama Disease:A Classic and Destructive Disease of Banana. *Plant Health*

Progress: 1–7.

Richey MG. **1989**. Transformation of Filamentous Fungi with Plasmid DNA by Electroporation. *Phytopathology* **79**: 844.

Robatzek S, Bittel P, Chinchilla D, Köchner P, Felix G, Shiu S-H, Boller T. **2007**. Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of Arabidopsis FLS2 exhibiting characteristically different perception specificities. *Plant Molecular Biology* **64**: 539–547.

Rohe M, Gierlich A, Hermann H, Hahn M, Schmidt B, Rosahl S, Knogge W. **1995**. The race-specific elicitor, NIP1, from the barley pathogen, Rhynchosporium secalis, determines avirulence on host plants of the Rrs1 resistance genotype. *The EMBO Journal* **14**: 4168–77.

Rohe M, Searle J, Newton AC, Knogge W. **1996**. Transformation of the plant pathogenic fungus, Rhynchosporium secalis. *Current Genetics* **29**: 587–590.

Ron M, Avni A. **2004**. The Receptor for the Fungal Elicitor Ethylene-Inducing Xylanase Is a Member of a Resistance- Like Gene Family in Tomato Author (s): Mily Ron and Adi Avni Published by : American Society of Plant Biologists (ASPB) Stable URL : http://www.jstor.org/stab. *The Plant Cell* **16**: 1604–1615.

Rooney HCE, van 't Klooster JW, Van der Hoorn RAL, Joosten MHAJ, Jones JD, De Wit PJGM. **2005**. Cladosporium Avr2 Inhibits Tomato Rcr3 Protease Required for Cf-2-Dependent Disease Resistance. *Science* **308**: 1783–1786.

Rowland O, Ludwig A a, Merrick CJ, Baillieul F, Tracy FE, Yoshioka H, Durrant WE, Fritz-laylin L, Nekrasov V, Sjo K, *et al.* 2005. Functional Analysis of Avr9 / Cf-9 Rapidly Elicited Genes Identifies a Protein Kinase , ACIK1 , That Is Essential for Full Cf-9 – Dependent Disease Resistance in Tomato. *The Plant cell* **17**: 295–310.

Salomon D, Bonshtien A, Mayrose M, Zhang C, Shokat KM, Sessa G. **2009**. Bypassing Kinase Activity of the Tomato Pto Resistance Protein with Small Molecule Ligands. *Journal of Biological Chemistry* **284**: 15289–15298.

Saur IML, Kadota Y, Sklenar J, Holton NJ, Smakowska E, Belkhadir Y, Zipfel C, Rathjen JP. **2016**. NbCSPR underlies age-dependent immune responses to bacterial cold shock protein in Nicotiana benthamiana. *Proceedings of the National Academy of Sciences of the United States of America* **113**: 1511847113-.

Schmidt SM, Kuhn H, Micali C, Liller C, Kwaaitaal M, Panstruga R. **2014**. Interaction of a Blumeria graminis f. sp. hordei effector candidate with a barley ARF-GAP suggests that host vesicle trafficking is a fungal pathogenicity target. *Molecular Plant Pathology* **15**: 535–549.

Schornack S, van Damme M, Bozkurt TO, Cano LM, Smoker M, Thines M, Gaulin E, Kamoun S, Huitema E. **2010**. Ancient class of translocated oomycete effectors targets the host nucleus. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 17421–17426.

Schürch S, Linde CC, Knogge W, Jackson LF, McDonald BA. **2004**. Molecular Population Genetic Analysis Differentiates Two Virulence Mechanisms of the Fungal Avirulence Gene NIP1. *Molecular Plant-Microbe Interactions* **17**: 1114–1125.

Schweizer P, Stein N. **2011**. Large-Scale Data Integration Reveals Colocalization of Gene Functional Groups with Meta-QTL for Multiple Disease Resistance in Barley. *Molecular Plant-Microbe Interactions* **24**: 1492–1501.

Segonzac C, Feike D, Gimenez-Ibanez S, Hann DR, Zipfel C, Rathjen JP. **2011**. Hierarchy and Roles of Pathogen-Associated Molecular Pattern-Induced Responses in Nicotiana benthamiana. *Plant Phisiology* **156**: 687–699.

Shah DM, Rommens CMT, Beachy RN. **1995**. Resistance to diseases and insects in transgenic plants: progress and applications to agriculture. *Trends in Biotechnology* **13**: 362–368.

Sham A, Al-Azzawi A, Al-Ameri S, Al-Mahmoud B, Awwad F, Al-Rawashdeh A, Iratni R, AbuQamar S. **2014**. Transcriptome analysis reveals genes commonly induced by Botrytis cinerea infection, cold, drought and oxidative stresses in Arabidopsis. *PLoS ONE* **9**: 1–26.

Shan L, He P, Li J, Heese A, Peck SC, Nürnberger T, Martin GB, Sheen J. **2008**. Bacterial Effectors Target the Common Signaling Partner BAK1 to Disrupt Multiple MAMP Receptor-Signaling Complexes and Impede Plant Immunity. *Cell Host & Microbe* **4**: 17–27.

Shan L, He P, Zhou JM, Tang X. **2000**. A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. *MPMI* **13**: 592–598.

Sharma SK, Bolser D, de Boer J, Sønderkær M, Amoros W, Carboni MF, D'Ambrosio JM, de la Cruz G, Di Genova A, Douches DS, *et al.* 2013. Construction of reference chromosome-scale pseudomolecules for potato: integrating the potato genome with genetic and physical maps. *G3 (Bethesda, Md.)* **3**: 2031–47.

Shiu S, Karlowski W, Pan R. **2004**. Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *The Plant Cell …* **16**: 1220–1234.

Simpson AJG, Reinach FC, Arruda P, Abreu FA, Acencio M, Alvarenga R, Alves LMC, Araya JE, Baia GS, Baptista CS, *et al.* 2000. The genome sequence of the plant pathogen Xylella fastidiosa. *Nature* **406**: 151–157.

Song W, Wang G, Chen L, Kim H, Pi L, Hoisten T, Gardner J, Wang B, Zhai W, Zhu L, *et al.*

1995. A Receptor Kinase-Like Protein Encoded by the Rice Disease Resistance Gene, Xa21. *Science*: 2–4.

Southern J a, Young DF, Heaney F, Baumgartner WK, Randall RE. **1991**. Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. *Journal of General Virology* **72**: 1551–1557.

Sperschneider J, Dodds PN, Gardiner DM, Manners JM, Singh KB, Taylor JM. **2015**. Advances and Challenges in Computational Prediction of Effectors from Plant Pathogenic Fungi (DC Sheppard, Ed.). *PLOS Pathogens* **11**: e1004806.

Spoel SH. **2003**. NPR1 Modulates Cross-Talk between Salicylate- and Jasmonate-Dependent Defense Pathways through a Novel Function in the Cytosol. *The Plant cell* **15**: 760–770.

Spoel SH, Johnson JS, Dong X. **2007**. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proceedings of the National Academy of Sciences* **104**: 18842–18847.

Stakman. **1915**. Relation between Puccinia Graminis and plants hihgly resistant to its attack. *Journal of Agricultural Research* **IV**: 1–4.

Staskawicz BJ, Dahlbeck D, Keen NT. **1984**. Cloned avirulence gene of *Pseudomonas syringae pv. glycinea* determines race-specific incompatibility on Glycine max (L.) Merr. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 6024–6028.

Steiner-Lange S, Fischer A, Boettcher A, Rouhara I, Liedgens H, Schmelzer E, Knogge W. **2003**. Differential Defense Reactions in Leaf Tissues of Barley in Response to Infection by Rhynchosporium secalis and to Treatment with a Fungal Avirulence Gene Product. *Molecular Plant-Microbe Interactions* **16**: 893–902.

Stergiopoulos I, de Wit PJ. **2009**. Fungal effector proteins. *Annu Rev Phytopathol* **47**: 233–263.

Strange RN, Scott PR. **2005**. Plant Disease: A Threat to Global Food Security. *Annual Review of Phytopathology* **43**: 83–116.

Sun W, Dunning FM, Pfund C, Weingarten R, Bent AF. **2006**. Within-species flagellin polymorphism in Xanthomonas campestris pv campestris and its impact on elicitation of Arabidopsis FLAGELLIN SENSING2-dependent defenses. *The Plant cell* **18**: 764–79.

Sundstrom P. **2002**. Adhesion in Candida spp. *Cellular Microbiology* **4**: 461–469.

Sundström JF, Albihn A, Boqvist S, Ljungvall K, Marstorp H, Martiin C, Nyberg K, Vågsholm I, Yuen J, Magnusson U. **2014**. Future threats to agricultural food production posed by environmental degradation, climate change, and animal and plant diseases – a risk analysis in three economic and

climate settings. *Food Security* **6**: 201–215.

Taggart PJ, Cooke LR, Mercer PC, Shaw MW. **1998**. Effects of fungicides used to control Rhynchosporium secalis where benzimidazole resistance is present. *Crop Protection* **17**: 727–734.

Taggart PJ, Locke T, Phillips AN, Pask N, Hollomon DW, Kendall SJ, Cooke LR, Mercer PC. **1999**. Benzimidazole resistance in Rhynchosporium secalis and its effect on barley leaf blotch control in the UK. *Crop Protection* **18**: 239–243.

Taguchi F, Takeuchi K, Katoh E, Murata K, Suzuki T, Marutani M, Kawasaki T, Eguchi M, Katoh S, Kaku H, *et al.* 2006. Identification of glycosylation genes and glycosylated amino acids of flagellin in Pseudomonas syringae pv. tabaci. *Cellular Microbiology* **8**: 923–938.

Taguchi F, Yamamoto M, Ohnishi-Kameyama M, Iwaki M, Yoshida M, Ishii T, Konishi T, Ichinose Y. **2010**. Defects in flagellin glycosylation affect the virulence of Pseudomonas syringae pv. tabaci 6605. *Microbiology* **156**: 72–80.

Takahashi A, Kawasaki T, Henmi K, Shii K, Kodama O, Satoh H, Shimamoto K. **1999**. Lesion mimic mutants of rice with alterations in early signaling events of defense. *Plant Journal* **17**: 535–545.

Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y, Shinohara A, Takeda S. **1998**. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *The EMBO Journal* **17**: 5497–5508.

Talbot NJ. **2003**. On the trail of a cereal killer: Exploring the biology of Magnaporthe grisea. *Annual review of microbiology* **57**: 177–202.

Tamaoki D, Seo S, Yamada S, Kano A, Miyamoto A, Shishido H, Miyoshi S, Taniguchi S, Akimitsu K, Gomi K. **2013**. Psb-8-E24260. : 8–10.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. **2013**. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Thangudu RR, Manoharan M, Srinivasan N, Cadet F, Sowdhamini R, Offmann B. **2008**. Analysis on conservation of disulphide bonds and their structural features in homologous protein domain families. *BMC Structural Biology* **8**: 55.

Thatcher LF, Williams AH, Garg G, Buck S-AG, Singh KB. **2016**. Transcriptome analysis of the fungal pathogen Fusarium oxysporum f. sp. medicaginis during colonisation of resistant and susceptible Medicago truncatula hosts identifies differential pathogenicity profiles and novel candidate effectors. *BMC Genomics* **17**: 860.

Thirugnanasambandam A, Wright KM, Atkins SD, Whisson SC, Newton AC. **2011**. Infection of

Rrs1 barley by an incompatible race of the fungus Rhynchosporium secalis expressing the green fluorescent protein. *Plant Pathology* **60**: 513–521.

Thomma BPHJ, Van Esse HP, Crous PW, De Wit PJGM. **2005**. Cladosporium fulvum (syn. Passalora fulva), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Molecular Plant Pathology* **6**: 379–393.

Thomma BPHJ, Nürnberger T, Joosten MHAJ. **2011**. Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *The Plant cell* **23**: 4–15.

Thomma BPHJ, Penninckx IAMA, Broekaert WF, Cammue BPA. **2001**. The complexity of disease signaling in Arabidopsis. *Current Opinion in Immunology* **13**: 63–68.

Tor M. **2002**. Arabidopsis SGT1b Is Required for Defense Signaling Conferred by Several Downy Mildew Resistance Genes. *The Plant cell* **14**: 993–1003.

Tsuda K, Katagiri F. **2010**. Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Current Opinion in Plant Biology* **13**: 459–465.

Tyler BM. **2007**. Phytophthora sojae: Root rot pathogen of soybean and model oomycete. *Molecular Plant Pathology* **8**: 1–8.

De Vleesschauwer D, Xu J, Höfte M. **2014**. Making sense of hormone-mediated defense networking: from rice to Arabidopsis. *Frontiers in plant science* **5**: 611.

Walters DR, Avrova A, Bingham IJ, Burnett FJ, Fountaine J, Havis ND, Hoad SP, Hughes G, Looseley M, Oxley SJP, *et al.* 2012. Control of foliar diseases in barley: towards an integrated approach. *European Journal of Plant Pathology* **133**: 33–73.

Wan J, Zhang X-C, Neece D, Ramonell KM, Clough S, Kim S -y., Stacey MG, Stacey G. **2008**. A LysM Receptor-Like Kinase Plays a Critical Role in Chitin Signaling and Fungal Resistance in Arabidopsis. *The Plant cell* **20**: 471–481.

Wang Z, Gerstein M, Snyder M. **2009**. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* **10**: 57–63.

Wang W, Vinocur B, Altman A. **2003**. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* **218**: 1–14.

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux J-P, Ryals JA. **1991**. Coordinate Gene Activity in Response to Agents That lnduce Systemic Acquired Resistance. *The Plant Cell* **3**: 1085–1094.

van Wees SC, de Swart E a, van Pelt J a, van Loon LC, Pieterse CM. **2000**. Enhancement of

induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 8711–8716.

Wei Z, Laby RJ, Zumoff CH, Bauer DW, He SY, Colimer A, Beer S V. **1992**. Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen Erwinia amylovora. *Science* **321**: 85–88.

Weld RJ, Plummer KM, Carpenter MA, Ridgway HJ. **2006**. Approaches to functional genomics in filamentous fungi. *Cell Research* **16**: 31–44.

Wetlaufer DB. **1973**. Nucleation, Rapid Folding, and Globular Intrachain Regions in Proteins. *Pnas* **70**: 697–701.

Wevelsiep L, Kogel K-H, Knogge W. **1991**. Purification and characterization of peptides from Rhynchosporium secalis inducing necrosis in barley. *Physiological and Molecular Plant Pathology* **39**: 471–482.

Wevelsiep L, Rupping E, Knogge W. **1993**. Stimulation of Barley Plasmalemma H+-ATPase by Phytotoxic Peptides from the Fungal Pathogen Rhynchosporium secalis. *Plant Physiology* **101**: 297–301.

Whitman DB. **1999**. Genetically Modified Foods: Harmful or Helpful? *Nature* **399**: 11931–11936.

Williamson B, Tudzynski B, Tudzynski P, van Kan JAL. **2007**. Botrytis cinerea: the cause of grey mould disease. *Molecular Plant Pathology* **8**: 561–580.

Wulff BB, Horvath DM, Ward ER. **2011**. Improving immunity in crops: new tactics in an old game. *Current Opinion in Plant Biology* **14**: 468–476.

Xiang J, Li X, Wu J, Yin L, Zhang Y, Lu J. **2016**. Studying the mechanism of Plasmopara viticola RxLR effectors on suppressing plant immunity. *Frontiers in Microbiology* **7**: 1–12.

Xiang T, Zong N, Zou Y, Wu Y, Zhang J, Xing W, Li Y, Tang X, Zhu L, Chai J, *et al.* 2008. Pseudomonas syringae Effector AvrPto Blocks Innate Immunity by Targeting Receptor Kinases. *Current Biology* **18**: 74–80.

Xi K, Turkington TK, Helm JH, Bos C. **2002**. Pathogenic variation of Rhynchosporium secalis in Alberta. *Canadian Journal of Plant Pathology* **24**: 176–183.

Yang F, Li W, Jørgensen HJL. **2013**. Transcriptional Reprogramming of Wheat and the Hemibiotrophic Pathogen Septoria tritici during Two Phases of the Compatible Interaction. *PLoS ONE* **8**: 1–15.

Yang Y, Zhang H, Li G, Li W, Wang X, Song F. **2009**. Ectopic expression of MgSM1, a Cerato-

platanin family protein from Magnaporthe grisea , confers broad-spectrum disease resistance in Arabidopsis. *Plant Biotechnology Journal* **7**: 763–777.

Yuan C, Li C, Yan L, Jackson AO, Liu Z, Han C, Yu J, Li D. **2011**. A High Throughput Barley Stripe Mosaic Virus Vector for Virus Induced Gene Silencing in Monocots and Dicots (A Rahman, Ed.). *PLoS ONE* **6**: e26468.

Yu X, Tang J, Wang Q, Ye W, Tao K, Duan S, Lu C, Yang X, Dong S, Zheng X, *et al.* 2012. The RxLR effector Avh241 from Phytophthora sojae requires plasma membrane localization to induce plant cell death. *New Phytologist* **196**: 247–260.

Zadoks JC. **2008**. The potato murrain on the European continent and the revolutions of 1848. *Potato Research* **51**: 5–45.

Zaffarano PL, McDonald B a, Linde CC. **2011**. Two new species of Rhynchosporium. *Mycologia* **103**: 195–202.

Zaffarano PL, McDonald B a, Zala M, Linde CC. **2006**. Global Hierarchical Gene Diversity Analysis Suggests the Fertile Crescent Is Not the Center of Origin of the Barley Scald Pathogen Rhynchosporium secalis. *Phytopathology* **96**: 941–950.

Zeidler D, Zähringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P, Durner J. **2004**. Innate immunity in Arabidopsis thaliana: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 15811–15816.

Zhan J, Fitt BDL, Pinnschmidt HO, Oxley SJP, Newton AC. **2008**. Resistance, epidemiology and sustainable management of Rhynchosporium secalis populations on barley. *Plant Pathology* **57**: 070918211612002–???

Zhan J, Yang L, Zhu W, Shang L, Newton AC. **2012**. Pathogen Populations Evolve to Greater Race Complexity in Agricultural Systems – Evidence from Analysis of Rhynchosporium secalis Virulence Data (D Arnold, Ed.). *PLoS ONE* **7**: e38611.

Zhang W, Fraiture M, Kolb D, Loffelhardt B, Desaki Y, Boutrot FFG, Tor M, Zipfel C, Gust A a, Brunner F. **2013**. Arabidopsis RECEPTOR-LIKE PROTEIN30 and Receptor-Like Kinase SUPPRESSOR OF BIR1-1/EVERSHED Mediate Innate Immunity to Necrotrophic Fungi. *The Plant Cell* **25**: 4227–4241.

Zhang L, Kars I, Essenstam B, Liebrand TWH, Wagemakers L, Elberse J, Tagkalaki P, Tjoitang D, van den Ackerveken G, van Kan JAL. **2014**. Fungal Endopolygalacturonases Are Recognized as Microbe-Associated Molecular Patterns by the Arabidopsis Receptor-Like Protein RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1. *Plant Physiology* **164**: 352–

364.

Zhang Q, Webster RK, Crandall BA, Jackson LF, Maroof MAS. **1992**. Race Composition and Pathogenicity Associations of Rhynchosporium secalis in California. *Phytopathology* **82**: 798–803.

Zhang J, Zhou J-M. **2010**. Plant Immunity Triggered by Microbial Molecular Signatures. *Molecular Plant* **3**: 783–793.

Zheng X, McLellan H, Fraiture M, Liu X, Boevink PC, Gilroy EM, Chen Y, Kandel K, Sessa G, Birch PRJ, *et al.* 2014. Functionally Redundant RXLR Effectors from Phytophthora infestans Act at Different Steps to Suppress Early flg22-Triggered Immunity. *PLoS Pathogens* **10**.

Zhou J, Tang X, B. Martin G. **1997**. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *The EMBO Journal* **16**: 3207–3218.

Zipfel C. **2014**. Plant pattern-recognition receptors. *Trends in Immunology* **35**: 345–351.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G. **2006**. Perception of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts Agrobacterium-Mediated Transformation. *Cell* **125**: 749–760.

Zipfel C, Robatzek S. **2010**. Pathogen-Associated Molecular Pattern-Triggered Immunity: Veni, Vidi...? *Plant Physiology* **154**: 551–554.

Zvelebil, M, Pluciennik, M. 2003. Historical Origins of Agriculture. In Squires, V. (ed.). E*ncyclopedia of Life Support Systems. The Role of Food, Agriculture, Forestry and Fisheries in Human Nutrition*. Vol. 1. Encyclopedia of Life Support Systems (EOLSS) Publishers LTD, Oxford.